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(Eds.)

Communication in Plants

Neuronal Aspects of Plant Life

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František Baluška · Stefano Mancuso · Dieter Volkmann (Eds.)

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Preface

As we enter the new millennium, plant biology is witnessing dramatic advancements in studies related to the complex behaviour of higher plants which are now beginning to reveal intelligent behaviour. Surprisingly, it is plant ecology which is leading in the revelation that plants behave as though having conscious comprehension of themselves and of their environment. Charles Darwin was the first who noted the abilities of plants to communicate with their environment and translate this information into active movements of their organs (Darwin 1880).

Plants recognize other organisms such as bacteria, fungi, other plants, insects, birds, and animals that presumably also include us, humans (Takabayashi and Dicke 1996; Paré and Tumlinson 1999; Kessler and Baldwin 2001). For instance, to accomplish their sexual reproduction, plants rely on complex interactions with insects and birds. In order to achieve this, and as Charles Darwin was one of the first to show (Darwin 1862), plants generate specially shaped sexual organs which allow insects and birds access to their flowers. Moreover, plants reward these pollinators with nectar and other compounds which are both attractive and a necessary part of the diet of these insect/bird feeders (Cozzolino and Widmer 2005). Complex interactions have been recorded between insect pheromones and plant volatile semiochemicals (Reddy and Guerrero 2004). In the case of many *Arum* spp., the insect-attracting plant volatiles with a dung-like odour are exactly those chemicals which attract insects to animal dung where they would otherwise gather and reproduce (Kite et al. 1998). These plants are thus masters of a deceptive and intelligent strategy for their own reproduction. Moreover, plants appear to possess an innate type of immunity system which closely resembles that of animals (Nürnberger et al. 2004) and, interestingly in this respect, there are also several parallels between the recognition of self and non-self in plant breeding systems and histocompatibility in animals (Nasrallah 2005). Plant roots of *Fabaceae* can recognize and 'domesticate' *Rhizobium* bacteria within nodules, and the composite bacteroids then supply the host plants with nitrogen. Less well known, perhaps, is that some plants recognize and communicate with ants (and vice versa) which protect them against herbivores, pathogens as well as competing vegetation (Brouat et al. 2001; Dejean et al. 2005). The plants, in

turn, reward the ants by secreting nectar (Heil et al. 2005) and constructing special food bodies (Solano et al. 2005). Plants actively recognize the identity of herbivores and are then able to recruit their enemies (Arimura et al. 2005). For instance, plant roots attacked by insect predators release volatiles which then attract particular species of nematodes that kill these predators (Rasmann et al. 2005). In addition, by releasing volatiles into the aerial environment, plant shoots infected by pathogens inform their neighbouring plants about immanent danger and they can then increase their immunity against these pathogens (Paré and Tumlinson 1999; Reddy and Guerrero 2004). Intriguingly, the signature of released volatiles is characteristic for herbivore damage but is different from that resulting from a general wound response (Reddy and Guerrero 2004; Arimura et al. 2005). *Nicotiana attenuata* attacked by the hornworm, *Manduca sexta*, accumulates nicotine, which poisons acetylcholine receptors, and is thus toxic to those organisms which rely on neuromuscular junctions (Baldwin 2001). Interestingly in this respect, plants express neuronal acetylcholinesterase (Sagane et al. 2005) and use acetylcholine also for their neuronal-like cell–cell communication (Momonoki et al. 1998). Furthermore, during their phylogeny, plants can also switch from an autotrophic to a heterotrophic lifestyle – a feat which, in the case of parasitic or carnivorous plants, requires the active selection of suitable host/prey organisms (Albert et al. 1992).

Plants are extremely mechanosensitive. Their roots exhibit thigmotropism, which enables them to explore, with an animal-like curiosity, their environment in a continual search for water and solutes, and their shoots sometimes seek support by means of tendrils, assisted in this task by volatiles such as jasmonates. Root apices constantly monitor the numerous physical parameters of the soil and use this information in their search for better niches for survival and reproduction. In this behaviour, plant roots closely resemble fungi and, indeed, most roots enter symbiotic interactions with mycorrhizal fungi in order to increase their efficiency in obtaining critical ions such as phosphorus. In fact, roots might prove to be descendents of ancient fungi which, by entering into close association with their symbiotic photoautotrophs, have developed into heterotrophic roots – there are, after all, close resemblances between the anatomies and functions of apices of both rhizomorphs and roots (Botton and Dexheimer 1977) – while photoautotrophs have developed into the autotrophic shoots of the organisms now known as vascular plants (Atsatt 1988; Selosse and Le Tacon 1998; Heckman et al. 2001). This scenario is strongly supported by present-day pioneer colonizers such as lichens, which, just as was the case with early land plants (Yuan et al. 2005), are able to survive in even the most extreme of environmental conditions.

Literally, plants nourish the whole world. They intercept the light energy arriving on Earth from the sunbeams and transform it via energy-poor

inorganic compounds into energy-rich organic matter which then serves as the food for all heterotrophic organisms. Also, the gasoline which fuels many of Man's mechanical devices is of plant origin. Plants thus stand at the interface between a seemingly hostile and violent universe, and a fertile planet Earth teeming with life. We might postulate that if we could understand plants better, they could reveal to us something of the great mystery of life. Aristotle and his pupils were convinced that plants have complex inner life including thoughts, memories, dreams, and plans for the future. Unfortunately, our contemporary science considers plants rather as passive creatures to be exploited if discovered to be useful, and to be cleared away if not. However, their passivity – that is, their inability to change their location or to communicate via sounds – is only relative to the hyperactivity of human existence and the fleeting timescale of Man's artefacts. But the recent advances in ecology and phenomenology outlined above urge a change in this biased perception of higher plants.

We should also remember that action potentials, the very characteristic and rapidest way of neuronal communication, were discovered in plants in 1873 (Davies 2004). In those early days, the cellular basis of animal brains was not accepted and the neuronal processes in brains were just starting to be explored. Since then, a large amount of data has been accumulated on electric phenomena in plants (Meylan 1971; Davies 2004). Currently, new exciting discoveries are revealing that electrical signals modulate and control such basic physiological processes in plants as photosynthesis and phototropism (Koziolek et al. 2004; Volkov 2005). Unfortunately, the mainstream of plant biology has never completely accepted plant electrophysiology, so this field has survived in a quasi-dormant state up until now when exciting advances in plant biology are allowing the introduction of plant neurobiology as a newly emerging field of plant sciences. One foundation of this new science is the discovery that not only do plant cells express diverse neuronal molecules but that they also communicate together via plant synapses (Baluška et al. 2005).

These glimpses of the fascinating and breathtaking complexity of plants raise urgent questions which will dominate the whole field of plant biology in the next decades. In particular: Do plants have some type of neuronal system which resembles that which underlies the behaviour of animals? Conversely, if plants turn out to be 'brain-less', then the question will emerge where and how do they store and process the information which they obtain about both the abiotic and biotic environments, and how do they then use this information to optimize their future behaviour? Do plants feel (as suggested by Aristotle) and experience pain? Further: Do plants hear, and can they perceive odours? The truth is that we do not know, although their extreme sensitivity to mechanical vibrations indicates that they can perceive voices and their responses to volatile gases suggest they have a type

of olfactory response. Importantly, our lack of knowledge should not justify claims that plants do not possess these abilities and properties. In fact, their complex, rational, and surely intelligent behaviour suggests just the opposite. This is why we should be more sensitive to these issues and should commence a serious enquiry into these urgent questions, utilizing minds trained in the 'scientific method' but which can also clearly differentiate between speculation and hypothesis (Huszagh and Infante 1989).

Finally, is it by chance that the Greek word 'neuron' refers to vegetable fibre? In fact, this happy and synchronistic coincidence might be taken to signify that the term plant neurobiology is fully justified! This book brings together all these new plant neuronal aspects and combines them with the classical plant electrophysiology. Plant neurobiology is commencing its emergence as a coherent science.

All the chapters of this volume were presented on the First Symposium on Plant Neurobiology, Florence (Italy), 17–20 May 2005. This Symposium was generously supported by Ente Cassa di Risparmio di Firenze. Editors would like to express their gratitude for this support.

The bank is also giving us money to buy 100 copies of the book (which is part of the deal with Springer), so for us it is absolutely essential to have this in this book.

Bonn, Bristol and Florence,
July 2005

František Baluška, Peter W. Barlow,
Stefano Mancuso and Dieter Volkmann

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1 The Green Plant as an Intelligent Organism

Anthony Trewavas

Abstract Intelligence is an aspect of complex adaptive behaviour and a term not normally applied to plants. This chapter indicates a change in concept is long overdue and if poets can recognize it (above) so should scientists. Networks that control information flow are described as intelligent and such networks exist in all single living cells and in more complex multicellular organisms. Phosphoneural bacterial networks are briefly considered and these exist in a slightly different molecular but more complex form in higher plant and animal cells. Intelligent behaviour involves the whole organism and such integration involves complex communication. Evidence that plants forage and act intelligently in acquiring resources is indicated. The phenotype is actively (not passively) constructed in response to a complex changing environment by decisions that best secure the well-being of the individual plant within the life cycle goal of optimal fitness.

More and more I have come to admire resilience Not the simple resistance of a pillow whose foam Returns over and over to the same shape but the sinuous Tenacity of a tree: finding the light newly blocked on one side It turns to another. A blind intelligence true But out of such persistence arose turtles, rivers, Mitochondria figs-all this resinous un-retractable earth.

Jane Hirshfield (2005)

1.1 Introduction

Intelligence is an aspect of adaptive behaviour, even in humans. Organisms that live in challenging but variable and competitive circumstances require forms of behaviour that rise to that challenge and must be equally flexible to improve fitness. Those best able to master their environment are those most likely to succeed in the Darwinian wars. "The success of a species depends on it performing well (surviving and producing offspring, i.e. fitness) in its own particular environment. And intelligence plays a critical part in this success." Warwick 2001, p. 9). Since the life cycle is probably a primary target of natural selection (McNamara and Houston 1996; Schlichting and Pigliucci 1998), efficient acquisition of necessary food resources during growth and development is an important aspect of subsequent fitness because there is a common relation between accumulated resources and subsequent sibling number (Bazzaz 1996).

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1.1.1
The Problems of Subjective Intelligence

Before embarking on a discussion of plant intelligence it is essential to indicate what is meant by the term. The actual word is derived from the Latin *inter legere* meaning simply to choose. Dictionary definitions of intelligence use terms such as self-recognition or capacity for understanding and are couched in human terms. These definitions are perfectly adequate for public discussion that usually only involves human beings. But for biologists who wish to investigate and understand intelligence in other organisms such definitions lack useful substance.

A common problem is subjective intelligence. For example the cyberneticist, Warwick (2001, p. 9) states that "Comparisons (of intelligence) are usually made between characteristics that humans consider important; such a stance if of course biased and subjective in terms of the groups for whom it is being used." And as he shows is easily discredited. "When we compare the important aspects of intelligence, it is those which allow one species to dominate and exert power over other species that are of prime importance" (Warwick 2001). Bearing in mind the fact that plants dominate the planet, this statement is of importance for understanding plant intelligence. A further common assumption is that only organisms with brains (primates, cetaceans, crows) can be intelligent. Vertosick (2002) describes this as simple "brain chauvinism" and Schull (1990) goes further in stating that such views ascribe nerve cells as having some sort of vitalistic quality.

1.1.2
An Ability to Integrate a Multiplicity of Information into a Response Is an Important Intelligent Capability

Plants and animals are not passive objects in the face of environmental disturbance as indicated in the poem by Hirshfield (2005). They react and positively fashion themselves according to the information (signals) being received. Behaviour is the response to signals (Silvertown and Gordon 1989). Animals move when signalled, plants change their phenotype (Trewavas 2003). After that information is processed and integrated with the internal information, a response is constructed that improves fitness, the ultimate goal.

Green plants respond to numerous environmental biotic factors such as food resources (light, minerals, water) mechanical stimuli, humidity, soil structure, temperature and gases (Trewavas 2000; Turkington and Aarsen 1984). In each case the strength, direction, specific characteristics (e.g.

light wavelength) and intensity can be separately discriminated (Ballare 1994, 1999), and further complexity is added by virtue of the availability of resources being present either in fluctuating quantities varying from seconds to months, gradients with fluctuating intensity or a mosaic in the soil of vastly different concentrations (Bell and Lechowicz 1994; Farley and Fitter 1999; Grime 1994; Kuppers 1994; Pearcy et al. 1994; Robertson and Gross 1994) and others. Biotic signals are also sensed and acted upon and these include space; presence, absence and identity of neighbours (Tremmel and Bazzaz 1993); disturbance; competition (Darwinkel 1978; Goldberg and Barton 1992; Tremmel and Bazzaz 1995), predation and disease (Callaway et al. 2003; Turkington and Aarsen 1984). We understand little of the nature of the signals involved. Growth of individuals and neighbours continually and specifically changes the information spectrum.

There is no unique separate response to each signal in this complex but merely a response issued from an integration of all environmental and internal information. In the case of green plants, the visible response to signals is phenotypic plasticity (Bradshaw 1965; Schlichting and Pigliucci 1998; Sultan 2000). During information processing all signals meet somewhere in the cellular and tissue reactions that specify changes in form.

In seeking to understand the biological origins of human intelligence, Stenhouse (1974) described intelligence as adaptively variable behaviour during the lifetime of the individual in an attempt to discriminate intelligent behaviour from autonomic, that is unvarying, responses. Given the plethora of signals that plants integrate into a response, autonomic responses do not occur. Signal perception is instead ranked according to assessments of strength and exposure. But autonomic responses can be rejected; the numbers of different environments that any wild plant experiences must be almost infinite in number. Only complex computation can fashion the optimal fitness response.

1.1.3 Experimental Circumstances Can Be Misleading

When one factor is experimentally varied at a time in an attempt to simplify the complexity that wild plants normally experience, all those factors that do not vary are still sensed and integrated with the modified variable. For example, exposing a dicot seedling root to a gravitational signal leads to the textbook response of a resumption of vertical growth. But gradients of humidity, minerals, light, temperature imposed in a different direction or touch can override the gravity signal (Eapen et al. 2003; Massa and Gilroy 2003). Further complexity can result from an individuality in response to any one imposed signal (Trewavas 1998). Again for example with

gravity, the growth trajectories with which each root approaches the vertical can be individual (Bennett-Clerk and Ball 1951, referenced in Trewavas 2003).

The common use of statistics to obliterate individual variation leads to assumptions that the response to signals is always replicable. If the same signal and response are chosen, the same genotype, the environmental conditions are identical and the results are averaged statistically, this is no doubt true (but then the same can be said of an IQ test for human beings). No such simplicity of circumstance is available to an individual wild plant, which in meeting an almost infinite variety of environmental states must construct individual responses to improve its own fitness. No genome could contain the information that would provide an autonomic response to every environmental state. And even cloned individuals do not exhibit identical responses.

However, it is not just abiotic factors that are critical. Natural selection operates on individuals and Darwin (1859) considered that there is "a deeply seated error of considering the physical conditions of a country as the most important for its inhabitants whereas it cannot be disputed that the nature of other inhabitants with which each one has to compete is generally a far more important element of success." Considering the number of different species and individuals that co-exist, each one variable in phenotype and characteristics, any individual plant faces complexity not simplicity. Instead we are left only with the possibility of non-heritable (epigenetic) means whereby optimal fitness is achieved. Plants adequately meet the Stenhouse (1974) definition of intelligence.

1.2 Intelligent Behaviour of Single Cells

1.2.1 Molecular Networks in Single Eucaryote Cells

Cells are organized structures and vital properties result from the connections between the molecular constituents of which they are composed (Kitano 2002; Trewavas 1998). Numerous molecular connections integrate into a higher emergent organized order that we recognize as living. It is now known (1) that various steps in metabolism act like many Boolean computer logic gates such as AND, OR and NOR (Bray 1995) and are termed chemical neurons (Arkin and Ross 1994; Hjelmfelt and Ross 1992; Okamoto et al. 1987), (2) that these chemical neurons can act as pattern-recognition systems (Hjelmfeldt et al. 1993), (3) that proteins can act as computational elements (Bray 1995), and (4) that protein phosphorylation using about

1,000 protein kinases in both animals and plants provides for enormous numbers of complex elements of control, switching mechanisms and including both complex positive and negative feedback interactions (Bhalla et al. 2002; Chock and Stadtman 1977; Ingolis and Murray 2002). Such chemical systems parallel the capabilities of simple neural network structures as a set of on/off switches with feedback (Hopfield 1982; Hopfield and Tank 1986) on which they are modelled (Hjelmfeldt et al. 1991, 1992). Even in simple networks collective computational properties arose with parallel processing and extensive numbers of associative memories emerged as attractors occupying part of the network. Chemical neurons and neural network behaviour have most applicability to signal transduction studies (Bray 2003).

From an alternative direction, use of phage display or two hybrid methods has shown that that all proteins participate in the cellular network, a structure composed of hubs and connectors in which the number of connections to any one protein obeys a simple power law (Bray 2003; Gavin et al. 2002; Maslov and Sneppen 2002; Ravasz et al. 2002; Tong et al. 2002). The metabolic and signalling networks are modular with recognizable recurring circuit elements or network motifs that (1) filter out spurious input fluctuation, (2) generate temporal patterns of expression, and (3) accelerate throughput (Alon 2003). Such structures provide for robust behaviour that can also be fragile (Alon et al. 1999; Carlson and Doyle 2002) and exhibit highly optimized tolerance of variations in individual protein constituents (McAdams and Arkin 1999). "The cell in which zillions of molecular events occur at a time computes in parallel fashion" (Huang 2000), just like a brain. Robustness results from sharing control throughout the metabolic and signalling network with controlling steps determined by the environmental state (Strohmann 2000). Emerging network structures indicates how complex feedback controls operate (Davidson et al. 2002).

The cellular network perceives continual environmental variation through a multiplicity of receptors. Transduction in plants involves numerous second messengers and kinases enabling network information flow that may diverge, branch, converge, adapt, synergize and integrate through cross talk (Trewavas 2000). Such networks learn either by increasing the synthesis of particular constituents or by changing the affinity between particular network steps by post-translational modification (Trewavas 1999). Memory is simply the retention with time of the enhanced pathway of information flow and can be accessed by other pathways through cross talk (Taylor and McAinsh 2004). Cellular networks capable of these properties are entitled to be called intelligent and indeed form the basis of machine intelligence (Warwick 2001) and other forms of biological intelligence (Vertosick 2002).

1.2.2 Bacterial Intelligence and Phosphoneural Networks

Bacteria respond to many signals in their environment with adaptive responses designed to improve fitness (Hellingwerf 2005). The basic transduction mechanism for these signals involves phosphorylation of specific proteins with conserved regions on histidine and aspartate residues (Hellingwerf 2005) and other less common mechanisms in bacteria such as serine/threonine phosphorylation and quorum sensing systems (Park et al. 2003a,b). Very early on, analogies were drawn between the connections that phosphorylation enables between bacterial proteins and the connections between neurone dendrites in higher animal brains. This led to their description as a phosphoneural network (Hellingwerf et al. 1995). The properties of these networks include signal amplification, associative responses (cross talk) and memory effects. Subsequent investigation indicated learning (Hoffer et al. 2001) and the realization that these simple networks provide the individual bacterial cell with informed decisions (Bijlsma and Groisman 2003) in a rudimentary form of intelligence.

"This simplest of animals (bacteria) exhibits a prototypical centralized intelligence system that has the same essential design characteristics and problem solving logic as is evident in all animal intelligence systems including humans" (La Cerra 2003). "Some of the most fundamental features of brains such as sensory integration, memory, decision making and the control of behaviour can all be found in these simple organisms" (Allmann 1999).

Hellingwerf (2005) considers the crucial aspect of human intelligence is associative memory, i.e. to identify non-identical systems as being related. In bacterial networks this is simply cross talk after learning.

But La Cerra and Bingham (1998) came to a different conclusion of the basic element of bacterial intelligence from considerations of chemotaxis. "The *sine qua non* of behavioral intelligence systems is the capacity to predict the future; to model likely behavioral outcomes in the service of inclusive fitness." This model is retained in bacteria for only several seconds, the time taken for perception to alter the behaviour of the chemotactic rotor.

1.2.3 Observations of Eucaryote Single Cell Intelligence

Grasse (1977) has described remarkable non-heritable behaviour in single-celled amoebae (Arcella and Chaos). Arcella, for example, uses several cunning methods to return to its normal position after accidental inversion, to

deliberately corner motile food (infusoria) or to escape from impalement. Grasse (1977, p. 213) describes this behaviour as that which Haeckel called the psychological ability (i.e. purposive behaviour or intelligence) of the cell. "I dedicate these remarks to those who would simplify the properties of living things to the points of insignificance ... The observation of an animal in action in its proper environment remains an exercise essential to the biologist" (Grasse 1977), a statement of direct and pointed relevance to plant biologists. The plant biologist McClintock (1984) echoes the previous psychological sentiment in the following statement abstracted from her Nobel Prize acceptance speech: "A goal for the future would be to determine the extent of knowledge the cell has of itself and how it utilizes this knowledge in a thoughtful manner when challenged." Thoughtful can be equated with Grasse's (Haeckel's) psychological ability.

The slime mould *Physarum* has been presented with a maze of differing lengths with food at the end and always chose the shortest path, indicating an ability to optimize food gain whilst minimizing economy of effort (Nakagaki et al. 2000). The authors of this paper state "this remarkable process of cellular computation implies that cellular materials can show a primitive intelligence". Single cells have been observed to be capable of choice. Amoebae will prey on *Tetrahymena* but avoid *Copromonas* and if given the choice *Paramecium* prefers small ciliates to bacteria (Corning 2003).

1.3 Other Forms of Biological Intelligence

Social insects (termites, bees, ants) in colonies construct nest structures, minimal paths to food or adaptively change resource acquisition, behaviour described as swarm intelligence (Bonabeau et al. 2000; Bonabeau and Meyer 2001; Bonabeau and Theraulaz 2000; Franks et al. 2003; Seeley 1995). "Indeed it is not to much to say that a bee colony is capable of cognition in much the same way that a human being is. The colony gathers and continually updates diverse information about its surroundings combines this with information about its internal state and makes decisions that reconcile its well being with its environment" (Seeley and Levin 1987). Swarm intelligence owed its basis to the connections between the individual workers that form a network and changes in communication change the behaviour of the whole colony.

Immune intelligence-immune systems learn how to construct the best antibody, remember and predict future bacterial evolution (De Castro and Timmis 2002; Vertosick and Kelly 1992; Vertosick 2002) and intelligent genomes have been described briefly elsewhere (Thaler 1994). Intelligent genomes are equally found in plants (Trewavas 2005). Finally intelligent

species have been proposed and analysed in some detail. “Plant and animal species are information processing entities of such complexity, integration and adaptive competence that it may be scientifically fruitful to regard them as intelligent” (Schull 1990). Schull (1990) indicates analogies between learning and natural selection, memory with ecological niche, etc.

1.4 The Intelligence of Green Plants

“The tip of the root acts like the brain of one of the lower animals” (Darwin 1882).

Information processing, learning, memory, decision making, choice, predictive modelling, associative memory, sensory integration and control of behaviour are all aspects of biological intelligence. Information processing, decision making, associative memory, sensory integration and control of behaviour have already been mentioned in respect to plant cell signal transduction. Numerous examples of direct memory can be found in Desbiez et al. (1984, 1991), Jaffe and Shotwell (1980), Marx (2004), Trewavas (1999), Verdus et al. (1997) and references therein. Indeed since green plants are composed of millions of cells and the evidence indicates the intelligent capabilities of individual cells, intelligent responses of the whole plant are expected. Plant cell signal transduction uses a similar range of molecules for transduction as animals (Gilroy and Trewavas 2001).

Intelligence is a behavioural property of the whole organism and this requires integrated behaviour that is clearly evident (Hartnett and Bazzaz 1983, 1985; Turkington and Klein 1991; Turkington et al. 1991). “Plants have evolved an integrated complex of hormonal systems – a coordinated but non-centralised intelligence system that manages resources” (LaCerra and Bingham 2002). Communication is complex, involving proteins, nucleic acids, electrical communication and turgor information amongst many other signals (Trewavas 2002, 2005). For example, rootstocks affect numerous shoot characteristics when grafted and the root messages involve in part transfer of specific homeobox proteins (Kim et al. 2001). Behavioural changes in phenotype particularly in competition are constructed to optimize fitness and efficient foraging behaviour is crucial.

Peak et al. (2004) have described an alternative mechanism, patchiness of behaviour amongst groups of guard cells. Cooperative interactions amongst these patches leads eventually to synchronization and subsequent optimization of water relations of the leaf. Recognition of behaviourally discrete patches of plant cells has been made for some time (Trewavas 2003) and the mechanism has parallels with synchronization in a network of oscillators with distributed natural frequencies (Strogatz 2001).

1.4.1 Decisions and Choice in Plant Development

Plants actively forage for food resources by changing their architecture, physiology and phenotype (De Kroon and Hutchings 1995; Drew et al. 1975; Evans and Cain 1995; Grime et al. 1986; Grime 1994; Hutchings and De Kroon 1994; Slade and Hutchings 1987). When patches of rich resource are located either by roots or by shoots and occupation of resource receptors reaches critical levels, decisions are made to initiate enormous proliferation, thus greatly increasing the surface area of absorption of both energy minerals and water. Decisions are thus made continuously as plants grow, placing roots, shoots and leaves in optimal positions according to the abundance of perceived resources. Perhaps most crucial is that individual plants compete vigorously with each other for resources and the decisions are designed to improve fitness at the expense of others.

When given the choice between soil occupied by other plants and unoccupied soil the roots of those plants examined move their new proliferation into unoccupied soil and away from competitors (Gersani et al. 1998, 2001). When roots are made to touch roots of alien individuals (but not their own), the decision is made to cease growth (Callaway et al. 2003). Individual plants grown with the same level of resources but in a bigger soil volume grow much larger (McConnaghy and Bazzaz 1991, 1992; Schenk et al. 1999). This suggests that plants have mechanisms that sense their own root distribution and optimize the phenotype. Plants are territorial (Schenk et al. 1999); they minimize competition from their own roots and prefer unoccupied soil (Callaway et al. 2003; Huber-Sannwald et al. 1997; Mahall and Callaway 1992).

If individuals are forced to grow in the same soil volume, the root system proliferates in order to competitively sequester available root resources from other individuals but with a trade off in seed production (Gersani et al. 2001; Maina et al. 2002). Further convincing studies indicate that root systems are self-sensing (Falik et al. 2003; Gruntmann and Novoplansky 2004; Holzapfel and Alpert 2003), an important aspect of intelligent behaviour. When clones of the same plant are separated, within several weeks the root systems recognize each other as alien and proliferate accordingly. Plants assess and respond to local opportunities that will in the future benefit the whole plant (Falik et al. 2003).

Similar events take place in the shoot. Petioles and pulvini of many leaves orient the plane of leaf growth to that of the primary plane of incident sunlight and can move leaves out of this plane if light is too damagingly intense (De Kroon and Hutchings 1995; Muth and Bazzaz 2002a, b, 2003; Paladin 1918). Leaves of shoots are often placed to minimize self-shading (Honda and Fisher 1978; Yamada et al. 2000) just as roots are placed to minimize

competition from other plants. And when branches are fully overgrown the connecting vascular system is sealed, leading eventually to death and abscission (Franco 1986; Honkanen and Hanioja 1994; Henrikkson 2001).

1.4.2 Predictive Modelling to Improve Fitness

La Cerra and Bingham (1998) regard predictive modelling of behavioural outcomes in the service of inclusive fitness as the sine qua non of intelligent behaviour. Virtually all decisions made by plants are directed towards a future goal of optimal fitness. Roots and shoots growing along gradients of minerals or light are modelling a future that will subsequently increase resource acquisition if continued. Even when resource receptors are finally triggered and proliferation of leaves and roots is initiated, predictive modelling is in full force because new leaves and roots only become sources when nearly mature (Taiz and Zeiger 1998). Ackerly and Bazzaz (1995) observed that in canopy gaps both branch and leaf polarity were constructed to align with the primary orientation of diffuse light, again the product of assessing future resource capture. Both negative and positive feedback controls must operate to flesh out the predictive model. Experiments analysing the decisions to promote the growth (and acquisition of root resources) of well-placed branches at the expense of those less well placed concluded that the decisions were based on the speculatively expected future than the prevailing conditions (Novoplansky 1996, 2003; Novoplansky et al. 1989). The mayapple, a forest-floor perennial, takes decisions that determine future branch or flower formation years in advance (Geber et al. 1997). Many trees make similar decisions on flower production at least a year ahead. Perhaps the flower bud abscission in a colder spring observed in many fruit trees reflects a new reassessment of that past decision with present conditions.

The parasitical plant dodder exhibits a choice of host by rejecting many suitable ones. Furthermore in the earliest foraging contact of a suitable host, the future return of resources from the host is assessed within a few hours and energy investment in numbers of parasitical coils (and thus haustoria) is optimized (Kelly 1990, 1992). Using a variety of hosts Kelly (1990, 1992) showed that dodder fits the Charnov (1976) model, an analysis that shows how animals optimize their energy investment as against subsequent energy gain during foraging. Foraging in some other plants supports the Charnov model for plants (Gleeson and Fry 1997; Wijesinghe and Hutchings 1999). As mentioned earlier *Physarum* likewise optimizes energy investment for energy gain (Nakagaki et al. 2000), behaviour described as intelligent.

Future changes in resource availability are also predicted. Reflected far-red light from vegetation is used by many plants to predict likely future (not present) light competition and to initiate a variety of leaf and stem phenotypic alterations to avoid or ameliorate this situation (Aphalo and Ballare 1995; Ballare 1994; 1999; Novoplansky et al. 1990). Tendrils adjust their circumnutation pattern to position themselves to appropriate supports and can unwind if the decision turns out later to be poor (Baillaud 1962; Darwin 1882; Von Sachs 1879) The stilt palm moves out of shade by differential growth of prop roots (Trewavas 2003; 2004). When provided with water only once a year young trees eventually predict the water supply and synchronize their growth and development accordingly (Hellmeier et al. 1997).

1.4.3 Internal Assessment of Present State Before Phenotypic Change

A statement by Seeley and Levin (1987) discussing intelligent hive behaviour can be paraphrased for plants. "It is not too much to say that a plant is capable of cognition in much the same way that a human being is. The plant gathers and continually updates diverse information about its surroundings, combines this with information about its internal state and makes decisions that reconcile its well being with its environment." Examples of internal assessment are common. Thus, excessive cadmium, salt, osmotic stress, high or low temperatures or mechanical stress which normally kill can be subsequently resisted by pretreatments under milder conditions (Amzallag et al. 1990; Baker et al. 1985; Brown and Martin 1981; Henslow 1895; Laroche et al. 1992; Zhong and Dvorak 1995). Other examples include the degree of leaf abscission, senescence (Addicott 1982) or guard cell behaviour in water stress but determined by previous N status (Taiz and Zeiger 1998), interactions between N and light on shoot growth (Trewavas 1986), the degree to which root growth is enhanced under water deprivation dependent on light status (Bloom et al. 1985), or the different effects on branch growth according to whether one branch is shaded or the whole tree (Henrikkson 2001; Honkanen and Hanioja 1994).

1.5 Conclusions and Future Prospects

Plants exhibit the properties of intelligent behaviour described by biologists for other organisms and should consequently be regarded as intelligent too. Many plant biologists have a passive view of plant growth and development

in which the life cycle is played out with occasional periods of stress that simply slow it down (Aphalo and Bellare 1995). An excellent analogy of the alternative active view posed here is to be found in social insects (Trewavas 2005). Not only are there numerous exploratory trails or flights to find rich resources but, once discovered, changes in colony communication ensure numerous individuals (like proliferating leaves or branch roots) are actively employed in resource acquisition. The whole system benefits by the changes in foraging form. Bell (1984) has drawn analogies between plant branching and the foraging system of ants. The plant phenotype is constructed to benefit the whole organism using environmental signals that are internally assessed against current and previous experience. Competition is crucial; the poem by Hirshfield (2005) uses the term resilience, that is a strategy to deal with competition and to optimize the developing phenotype for maximal seed production. Describing plants as intelligent organisms is a conceptual change that indicates plants make dedicated active phenotypic decisions that improve accomplishment of the life cycle and fitness.

A common mistake is to judge plant behaviour in human terms. Warwick (2001), who warns against such thinking, makes the important point of judging intelligence within the framework of the capability of the organism. For plants, phenotypic change is the most relevant criterion but this needs more detailed future analysis than space allows here.

Finally, a major difficulty in recognizing intelligent behaviour in plants arises from an inability to assess root behaviour adequately. What is needed is a non-invasive method of imaging three-dimensional root distributions on a continuous basis. Various possibilities such as MRI or tomography or others need exploration. There have been a few attempts in the past (penetrating isotopes, slanting glass) but these are not very satisfactory. The ultimate goal should be instrumentation that can enable accurate, continuous, three-dimensional monitoring of tree root systems in the wild as well as much smaller plants. Current methods rely largely on the destructive procedures of exhumation. Only when the root system can be continually monitored will the intelligent integration of whole plant behaviour be properly revealed.

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2 Neurobiological View of Plants and Their Body Plan

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Abstract All principal metabolic biochemical pathways are conserved in animal and plant cells. Besides this, plants have been shown to be identical to animals from several other rather unexpected perspectives. For their reproduction, plants use identical sexual processes based on fusing sperm cells and oocytes. Next, plants attacked by pathogens develop immunity using processes and mechanisms corresponding to those operating in animals. Last, but not least, both animals and plants use the same molecules and pathways to drive their circadian rhythms. Currently, owing to the critical mass of new data which has accumulated, plant science has reached a crossroads culminating in the emergence of plant neurobiology as the most recent area of plant sciences. Plants perform complex information processing and use not only action potentials but also synaptic modes of cell–cell communication. Thus, the term 'plant neurobiology' appears to be justified. In fact, the word neuron was taken by animal neurobiologists from Greek, where the original meaning of this word is 'vegetal fibre'. Several surprises emerge when applying a 'neurobiological' perspective to illustrate how the plant tissues and the plant body are organized. Firstly, root apices are specialized not only for the uptake of nutrients but they also seem to support neuronal-like activities based on plant synapses. These synapses transport auxin via synaptic processes, suggesting that auxin is a plant-specific neurotransmitter. Altogether, root apices emerge as command centres and represent the anterior pole of the plant body. In accordance with this perspective, shoot apices act as the posterior pole. They are specialized for sexual reproduction and the excretion of metabolic products via hydathodes, trichomes, and stomata. Next, vascular elements allow the rapid spread of hydraulic signals and classical action potentials resembling nerves. As plants are capable of learning and they take decisions about their future activities according to the actual environmental conditions, it is obvious that they possess a complex apparatus for the storage and processing of information.

> *Life has always seemed to me like a plant that lives on its rhizome. Its true life is invisible, hidden in the rhizome. The part that appears above ground lasts only a single summer. Then it withers away – an ephemeral apparition. When we think of the unending growth and decay of life and civilisations, we cannot escape the impression of absolute nullity. Yet I have never lost a sense of something that lives and endures underneath the eternal flux. What we see is the blossom, which passes. The rhizome remains.*
>
> Carl Gustav Jung: *Memories, dreams, reflections*, Collins and Routlege & Kegan Paul, London, 1963. Translated from German into English by Richard and Clare Winston.

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2.1 Introduction

It was Aristotle and his students who made the first philosophical attempts to understand plants in their complexity. At this ancient time, the main interest for plants was limited to their usefulness in medicine. Much later, in the sixteenth century, the first attempts were made to understand the basic principles of structure and function of plants. At first, these studies were largely devoted to plant distribution, taxonomy, and morphology. Later, because of the technological advances resulting in the invention of the microscope and inspired by the earlier work on medicine, anatomy and cytology were added to the plant sciences curriculum. In fact, the cellular nature of animals and plants was elaborated first in plants (Hooke 1665, reviewed by Baluška et al. 2004a).

By the end of nineteenth century, it was realized that plants were even more similar to animals than had been thought hitherto. In fact, Huxley (1853) went so far as to say that "The plant is, then, an animal confined in a wooden case...". Advances in physiology helped confirm this, especially with regard to some of the basic physiological processes, such as respiration, digestion, and cell growth, where plants often provided the material of choice for experimental studies. In such circumstances, plant physiology was born; and it now dominates work in the plant sciences. Furthermore, a big surprise is that plants have been shown to be identical to animals from several rather unexpected perspectives. For their reproduction, plants use identical sexual processes based on the fusion between sperm cells and oocytes (Smyth 2005). Next, plants attacked by pathogens develop immunity using the same processes and mechanisms that operate in animals (Nürnberger et al. 2004). Last, but not least, animals and plants use the same molecules and pathways to drive their circadian rhythms (Cashmore 2003). Currently, plant science has reached another crossroad. A critical mass of new data has been accumulated which has culminated in the establishment of plant neurobiology as the most recent discipline of plant sciences.

Traditionally, plants are considered to be passive creatures mostly because, relative to the perception of man, they hardly move and make no noise. However, recent advances in plant sciences clearly reveal that plants are "intelligent" organisms capable of learning and taking decisions in relation to their environmental situation (Trewavas 2001, 2003). Plants are not just passive victims of circumstance but, rather, are active organisms which can identify their herbivores and actively recruit enemies of these herbivorous predators (Dicke and Sabelis 1988; van der Putten et al. 2001). For instance, maize roots attacked by larvae of *Diabrotica* beetle induce volatile compounds which recruit entomopathogenic nematodes which in turn kill this rootworm (Rasman et al. 2005). Moreover, plants use a battery

of volatile compounds not only for plant–insect, but also for plant–plant, communication. Some of these serve as chemical warning signals by being sensed by other plants in the vicinity of the area attacked (Dicke and Sabelis 1988; van der Putten et al. 2001; Bais et al. 2004; Weir et al. 2004).

It is obvious that the immobility of plants imposes different and, perhaps, greater pressures on them if they are to survive. Smart plants can memorize stressful environmental experiences, and can call upon this information to take decisions about their future activities (Goh et al. 2003). Moreover, not only have neuronal molecules been found in plants (reviewed by Baluška et al. 2004b), but plant synapses are also present which use the same vesicular recycling processes for cell–cell communication as neuronal synapses (Baluška et al. 2005a). Roots respond sensitively, via increases of cytoplasmic calcium, to glutamate, while other amino acids do not show this feature (Filleur et al. 2005). Root systems can identify self and non-self roots (Gruntman and Novoplansky 2004). Recent new views about consciousness and self-awareness, when considered as biological phenomena inseparable from adaptation and learning processes (Searle 1997, 2004; Koch 2004a, b), are compatible with the new neurobiologically oriented view of plants.

2.2 Root Apex as the Anterior Pole of the Plant Body

Classically, the plant body is considered to have an apical–basal axis of polarity settled during embryogenesis, with the shoot tip representing the apical pole, and the root tip the basal pole of the plant body (Jürgens 2001). But there are several anatomical and physiological aspects which are incompatible with this view of the plant body axis. Originally, this terminology was derived from plant embryology where roots are considered to develop at the so-called basal end of the embryo (Baluška et al. 2005a). Nevertheless, this apical–basal terminology does not have any justification as plant embryos do not align along the gravity vector as is the case of postembryonic plant bodies. With reference to gravity, a positive gravity response, with downward movement of root apices, could be regarded as an apical or anterior feature. On the other hand, a negative response could be a basal or posterior feature. Such a neurobiological view of the plant body offers a possibility to unify plants with other multicellular organisms by defining the anterior–posterior axis of the postembryonic plant body. This would be logical as postembryonic plant bodies are clearly polarized into the root apices specialized for movements and uptake of nutrients, which are characteristics of the anterior pole. This is opposed by the shoot apices specialized for determinate growth and subsequent transformation into sexual organs, which are characteristics of the posterior pole.

Although plants cannot physically move, active root growth allows exploration of soil niches for nutrition. This implies that root apices are not only sites of nutrient uptake but also sites of forward movement, both of which are attributes of anterior poles of multicellular organisms (Douglas et al. 2005; Barlow, this volume). Moreover, our preliminary data suggest that, in addition, root apices are specialized for neuronal-like activities based on plant synapses (Baluška et al. 2004b, 2005a). Interestingly in this respect, roots enter into symbiotic interactions with bacteria (Denison and Toby Kiers 2004) and mycorrhizal fungi (Vandenkoornhuyse et al. 2002). In fact, most free-living roots are part of a root-fungus commune (Brundrett 2002). Moreover, roots are special also with respect to nematode parasitism when these hijack both auxin transport and signalling pathways to transform root stele cells into giant feeding cells (Hutangura et al. 1999; Bird and Kaloshian 2003). All this suggests that the underground roots are more engaged in social activities that require self-awareness than the aboveground shoots.

In contrast to shoot apices, root apices assemble active synapses along distinctive cell files (Fig. 2.1), show a clear developmental zonation with a transition zone (discussed later), and execute complex patterns of polar

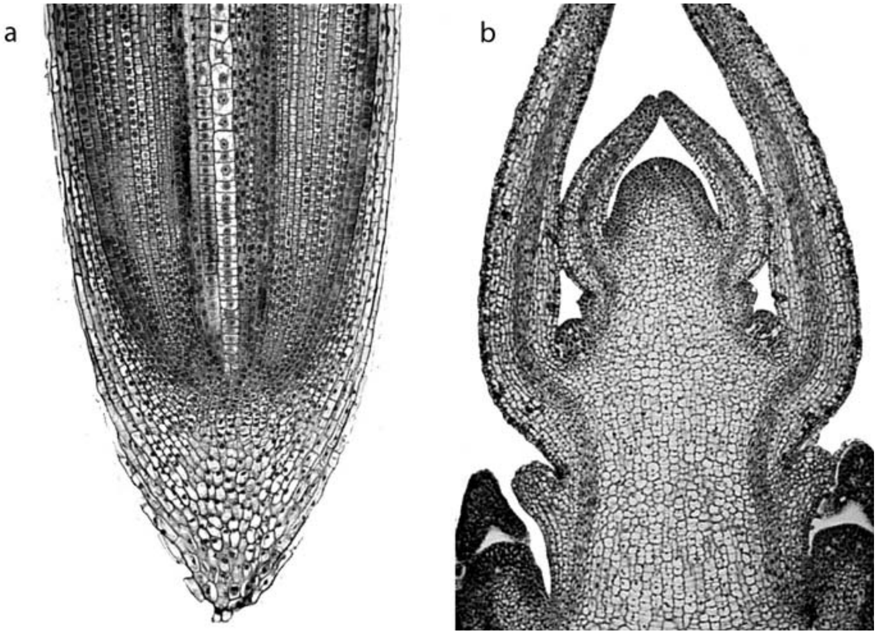

Fig. 2.1. Anatomical basis of root and shoot apices. Anatomical organization of root (**a**) and shoot (**b**) apices. Note very regular cell files, with cross-walls representing plant synapses, in root apices. On the other hand, cells in shoot apices are irregularly shaped and fail to arrange into regular cell files

auxin transport (Blilou et al. 2005; Kepinsky and Leyser 2005). On the other hand, shoots, bearing leaves and flowers, are more specialized to perform photosynthesis and sexual reproduction. Of course, flowers do entertain interactions with insects and even small birds (Raguso 2004), to allow effective spread of pollen, but flower cells do not interact directly with insect cells as is the case of root cells invaded by symbiotic bacteria and fungi. The latter act only as pathogens if they interact with shoots and leaves.

Parasitic plants provide very convincing evidence that roots represent the essential part of the plant, whereas shoots can be dispensable. If the plant nutrition is achieved by heterotrophic mechanisms then the plant is highly reduced to a haustorial system, derived from roots, specialized for organic nutrition. For instance, in holoparasitic plants, such as *Rafflesia*, the aboveground green part of the plant is completely missing (Brown 1822; Barkman et al. 2004). Nevertheless, haustoria of *Rafflesia* generate the largest flowers in the plant kingdom, which reveals that this unique organism really belongs to plants. Moreover, the primary role of roots in determining the nature of shoots is obvious also from grafting experiments which show that the rootstocks determine several shoot characteristics such as photosynthesis performance, shoot branching, leaf development, vein patterning, pathogen sensitivity, and stress tolerance (Jensen et al. 2003; Booker et al. 2004; Van Norman et al. 2004; Nelson 2004; Estan et al. 2005). Interestingly in this respect, non-pathogenic rhizobacteria interacting with roots can elicit induced systemic resistance in diverse plants against fungi, bacteria, and viruses (van Loon et al. 1998).

2.3
Shoot Apex as the Posterior Pole of the Plant Body

If the root apex is the anterior pole of the plant body then the shoot apex must represent the posterior pole. In all multicellular organisms, the posterior pole is specialized for excretion of metabolites and for sexual reproduction. Plants conform very well with this expectation. Their shoots harbour organs of excretion – the trichomes and hydathodes. Moreover, stomata perform gas exchange. Trichomes are unicellular or multicellular protuberances of shoot and leaf epidermis which allow removal of excess ions from the plant and can excrete toxic compounds via pores (striae) at their tips (Wagner et al. 2004; Kolb and Müller 2004). Trichomes also protect plants from herbivores, heat, and sunlight, and control leaf temperature and water loss, as well as regulating apoplasmic calcium (Fahn 2000; DeSilva et al. 2001; Jensen et al. 2003; Wagner et al. 2004; Kolb and Müller 2004). Interestingly, hydathodes seem to function analogously to the kidney (Pilot et al. 2004).

2.4 Auxin as a Plant Neurotransmitter

Auxin is the most important morphogenic agent shaping the whole plant body in accordance with two physical parameters – light and gravity (Friml 2003; Sachs 2004). Recently, we proposed that auxin represents a plant-specific neurotransmitter which is transported, in a light- and gravity-dependent manner, along the anterior–posterior axis of the plant body (Baluška et al. 2003a, b, 2004b, 2005a; Barlow et al. 2004). Importantly in this respect, auxin induces the formation of both vascular strands (plant nerves) as well as new root apices harbouring the command centre of the plant body (Baluška et al. 2004b). Root apices represent the major sink for polar auxin transport, and they also show extreme sensitivity to externally applied auxin (Jiang and Feldman 2002, 2005). Moreover, lateral root formation is induced by external auxin: initiation of root primordia is an endogenous process that recapitulates early embryogenesis (Jiang and Feldman 2002). In contrast, new shoots and leaves are formed exogenously from superficial cells.

2.5 Cellular End-Poles as Plant Synapses

Plant synapses are stable actin-supported adhesive domains, assembled at cellular end-poles (cross-walls) between adjacent plant cells of the same cell file, across which auxin and other chemical signals are transported from cell to cell via F-actin-driven and brefeldin A-sensitive vesicular trafficking pathways (Baluška et al. 2003a, b, 2004b, 2005a; Barlow et al. 2004). Besides these constitutive plant synapses, plants are also capable of forming facultative cell-to-cell junctions with cells of other organisms (plants, fungi, bacteria). These correspond to 'immunological synapses' (Baluška et al. 2005) – specialized cell-to-cell adhesion domains that involve the plasma membranes of the two organisms that are opposing each other. Such adhesive domains are also sites of active cell-to-cell transport of molecules and metabolites. Auxin-transporting plant synapses have been observed only in root apices where they are responsible for ordering of cells into very regular cell files (Baluška et al. 1997, 2000, 2003a, b, 2005a; see also Fig. 2.1a). In contrast, shoot apex cells do not align into such regular files (Fig. 2.1b) and resemble rather anatomically aberrant root apices of diverse mutants (Baluška et al. 2001a, 2003b) or after exposure of growing root apices to F-actin drugs such as latrunculin B (Baluška et al. 2001b).

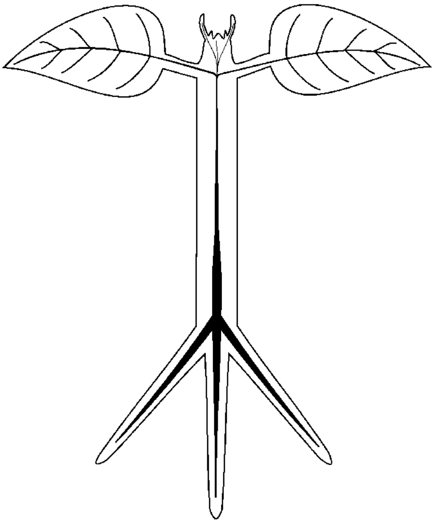

Fig. 2.2. Vascular bundles throughout the plant body. Thin strands of vascular tissue form networks in leaves, join into bundles in shoots, and transform into a large central cylinder of roots which is encircled by pericycle and endodermis

2.6 Vascular Strands as Plant Neurons

Vascular strands represent not only plant 'nerves' but also supply the plants with an endoskeleton. Along the plant body axis, there is a gradient of increasing volume and complexity of vascular/stelar tissues (Fig. 2.2). Leaves contain single thin strands which join together to form the vascular bundles. The latter extend along the stem up to the root to form the vascular cylinder (Sachs 2000). In roots, a large portion (up to 50% of the root diameter) of the organ is the vascular tissue, and its strands are supported by numerous 'nursery' cells forming the vascular cylinder (Sachs 2000). Moreover, stelar tissues in roots are completely enclosed by meristematic pericycle and protective endodermis. The latter tissue is ontogenetically related to the quiescent centre, while both endodermis and pericycle, like all vascular cells, are very active in transcellular auxin transport. Moreover, pericycle cells initiate lateral root formation in a process very closely resembling early zygotic events during embryo formation.

Phloem can be viewed as a supracellular axon-like 'channel' which connects the shoot and root apices. Phloem is specialized for transmission of action-potential-driven electric signals (Mancuso 1999). 'Axon-like' means that it is specialized for the rapid transfer of RNA molecules (Lucas et al. 2001) but it does not accomplish ribosome assembly and messenger

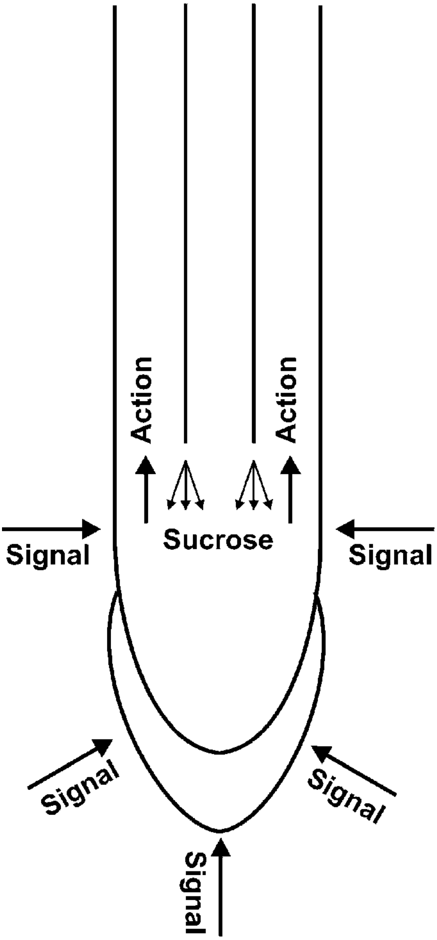

Fig. 2.3. Sensory zones in the root apex. There are two clearly defined sensory zones in the root apex: the root cap covering the meristem and the transition zone interpolated between the meristem and elongation region. Both these sensory zones receive diverse signals and the output is differential switch-like onset of rapid cell elongation, resulting in either straight growth (when all postmitotic cells start their rapid cell elongation simultaneously) or rapid turnings of the root apex. The transition zone is flooded with sucrose, which allows energy-demanding 'brain-like' information processing in its cells

RNA translation (van Bel 2003). Mature xylem elements represent non-living and water-filled tubes surrounded by metabolically active cells of xylem parenchyma (de Boer and Volkov 2003; Gilliham and Tester 2005). Xylem tubes are specialized for transmission of hydraulic signals, which are self-transmitting waves induced and driven by changes in hydrostatic pressure (Mancuso 1999).

2.7 Root Apices as "Brain-Like" Command Centres

Root apices show distinct cell files throughout their apices, a feature which is especially prominent in root apices with closed meristems, such as maize and *Arabidopsis*. The cell files are distinct because the end-poles of adjacent cells within cell files are tightly adhered, forming actin-based plant synapses transporting auxin (Baluška et al. 2003a, b, 2004b, 2005). In contrast, the shoot apices do not show distinct cells files and fail to show a DR5-signalled auxin maximum (Aloni et al. 2003) which is characteristic for root apices (Sabatini et al. 1999). Besides having synapses, root apices also exhibit clear zonation whereby the apical meristem joins to a so-called transition zone which has sensory capabilities and where root curvatures are initiated (Baluška et al. 2001c). As cells of the transition zone are not engaged in any demanding activities, such as mitotic divisions or rapid cell elongation, they are free to focus all their resources on the acquisition, processing, and storing of information. As they are close to the phloem unloading sites (Fig. 2.3), they are also flooded with sucrose (Stadler et al. 2005), which allows them to perform ATP-dependent processes such as robust ion channel activities, rapid vesicle recycling, and continuous cytoskeleton rearrangements. Interestingly, the transition zone phloem elements are of ancient type as they are lacking companion cells (van Bel 2003).

Postmitotic cells entering the transition zone reorganize actin filaments from a previously diffuse perinuclear network into robust bundles which extend from the nuclear surface towards non-growing end-poles (Baluška et al. 1997; Volkmann and Baluška 1999; Voigt et al. 2005). After reaching end-poles, the F-actin bundles anchor at these subcellular domains which are specialized for the synaptic vesicle recycling which drives transcellular auxin transport and which is also important for synaptic information processing and storing (Baluška et al. 2003b, 2004b, 2005). Another dramatic reorganization of the actin cytoskeleton is accomplished at the basal limit of the transition zone when conical actin bundles, organized around the centrally localized nucleus, become loosened, leave the nuclear surfaces, and extend longitudinally between the non-growing end-poles. This second reorganization of the actin cytoskeleton is essential for the onset of rapid cell elongation (Baluška et al. 1997; Volkmann and Baluška 1999; Baluška et al. 2000). In addition to the actin cytoskeleton, microtubules also undergo rearrangements in cells which traverse the transition zone in such a way that all cortical microtubules become transversely (with respect to the root axis) oriented. This allows polarization of cell expansion with rapidly extending side walls but non-growing end-poles (Baluška et al. 1992, 1993; Barlow and Baluška 2000).

Why does the transition zone exist? Why do root apices need a zone interpolated between the apical meristem and the elongation region which is almost the same size as the meristem? Root apices drive an exploratory mode of root growth in which the search is for oxygen, water, and ions to feed the whole plant body. This is not an easy task, and root apices have two zones which, in a coordinate fashion, allow them to perform rapid turnings. The first zone – transition zone – is close to the meristem and is the one most critical for the exploratory nature of root apices. Burst-like onset of rapid cell elongation, which can happen independently on the opposite root sides, allows instant turning of root apices (for graviresponse see Baluška et al. 1996). The second zone is the elongation region in which cell elongation can be slowed down differentially on the opposite root sides, thereby resulting in rapid turning of whole root apices (Massa and Gilroy 2003). In contrast, shoot apices lack clearly defined meristematic, transition, and elongation zones, and cannot perform such dynamic tropisms. Similarly, they lack regular cell files (Fig. 2.1) and presumably also very active synapses. Shoot apices cannot switch on differential rapid cell elongation, as root apices at the basal border of their transition zone, and the only mechanism is to change the growth rate of the cells. These so-called shade-avoidance shoot movements are much slower when compared with dynamic root behaviour.

Root apices are covered with a root cap (Barlow 2003) which protects the apex and also has numerous sensory abilities. It is a unique structure and is not present at the shoot apex. All this allows growing root apices to screen numerous environmental parameters, to process this information, and to change the growth direction accordingly. As a result, roots behave almost like more active animals, performing very efficient exploratory movements in their search for oxygen, water, and ions. Enclosed by the root cap is the quiescent centre, which represents the major catabolic sink for auxin (Jiang and Feldman 2002, 2005), and the apical portion of the root meristem, which is followed by the sensory transition zone (Baluška et al. 2001c). Furthermore, the distal portion of the transition zone represents the major sink both for exogenously applied auxin (Mancuso et al. 2005) and for oxygen while emitting large amounts of nitric oxide (Mancuso, Mugnai, Volkmann, Baluška, unpublished data). This anatomically distinct group of cells is unique in that it shows rhythmic patterns of ion fluxes and in this respect behaves as a brain-like organ (Baluška et al. 2004b).

Each root apex is proposed to harbour brain-like units of the nervous system of plants. The number of root apices in the plant body is high, and all 'brain units' are interconnected via vascular strands (plant neurons) with their polarly-transported auxin (plant neurotransmitter), to form a serial (parallel) neuronal system of plants. From observation of the plant body of maize, it is obvious that the number of root apices is extremely high,

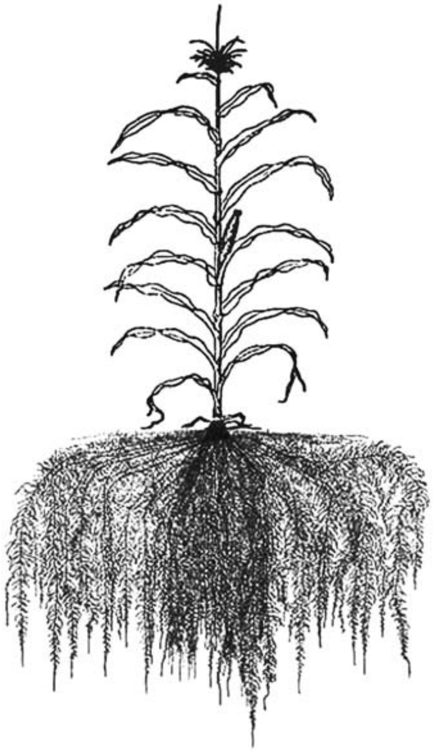

Fig. 2.4. Body plan of maize (*Zea mays*). Note the very rich root system within which there are numerous indeterminately growing root apices which are involved in inorganic nutrition for the whole plant and processing of neurobiological information (the anterior pole of the plant body). On the other hand, there are only few shoot apices with determinate growth which eventually transform into plant sexual organs (the posterior pole of the plant body)

whereas there are only few shoot apices (Fig. 2.4). This feature makes the 'serial plant brain' extremely robust and the amount of processed information must be immense.

2.8 Ancient Fungal-Like Nature of Roots

The most characteristic feature of roots is their invasive behaviour and exploratory nature based on their ability to actively penetrate soil in the permanent search for water and ions. In this respect, roots resemble fungi. Green shoots are less exploratory owing to the omnipresent light and specialization for photosynthesis. In holoparasitic plants like *Rafflesia*, the green part of the plant is missing completely, the root system being replaced

by an invasive 'fungus-like' network which penetrates the host tissues in a search for organic nutrition. In this respect, roots might represent fungal vestiges of putative ancient mergings between fungi and algae to generate higher plants (Atsatt 1988; Zyalalov 2004). This would explain the inherent tendency of roots to fuse with mycorrhizal fungi and thus generate plant root-fungal networks (Brundrett 2002). Early land plants suffered from a dry environment and could survive only if buried in wet parts of soil. After inventing a cuticle that would prevent desiccation, plants could extend into the aboveground space. Hypothetical merging between fungal and algal organisms would be beneficial for survival during this very critical period. Relevant in this respect is that lichens are also formed by association of fungal and algal partners. But this took place in recent times and the individual partners are still clearly distinguishable and separable. Recent advances in studies on lichens reveal that this association enables both components to invade separately hostile terrestrial niches exposed to high irradiation and dramatic desiccation (Kranner et al. 2005). It might be that similar, but much more ancient, associations between fungi and algae, resulting in the establishment of root-fungus communal networks, allowed plants to colonize land. The fungal/animal-like nature of roots is also supported by holoparasitic plants which can rely solely on the plant host which is penetrated via fungal-like root-derived haustoria (Yoder 2001). Parasitic plants represent an extremely diverse group of organisms with regard to their taxonomy and they have profound effects on the ecosystems in which they live (Press and Phoenix 2004).

Roots resemble ancient plants in their intimate association with water which is taken up and distributed throughout the plant body (Zyalalov 2004). The root apoplasm is freely accessible to the water which surrounds the root system. Despite the effective colonization of land, roots are still continuously bathed in aqueous soil solutions. Fungal features of roots are interesting also from the perspective of the inherent symbiotic interactions between roots and fungi (Vandenkoornhuyse et al. 2002). Whereas roots of most plant species are engaged in symbiotic interactions with fungi (Brundrett 2002; Karandashov and Bucher 2005), the aboveground organs do not share this feature and fungi are, by contrast, pathogens of shoots. The only cells of aboveground organs with a fungus-like characteristic are pollen tubes which are capable of an active lifestyle owing to their haustorium-like growth within female tissues (Palanivelu and Preuss 2000), resembling in this respect the root-derived haustoria of holoparasitic plants.

Another ancient root feature relates to their phloem elements. In root apices, protophloem lacks companion cells which are associated with all other phloem elements of angiosperm plants (van Bel 2003). In this respect, root protophloem resembles the conductive system of moss gametophytes.

2.9 Conclusions and Future Prospects

Our view of plants is changing dramatically, tending away from seeing them as passive entities subject to environmental forces and organisms that are designed solely for accumulation of photosynthetic products. The new view, by contrast, is that plants are dynamic and highly sensitive organisms, actively and competitively foraging for limited resources both above and below ground, and that they are also organisms which accurately compute their circumstances, use sophisticated cost–benefit analysis, and that take defined actions to mitigate and control diverse environmental insults. Moreover, plants are also capable of a refined recognition of self and non-self and this leads to territorial behaviour. This new view considers plants as information-processing organisms with complex communication throughout the individual plant. Plants are as sophisticated in behaviour as animals but their potential has been masked because it operates on time scales many orders of magnitude longer than that operating in animals.

Plants are sessile organisms. Owing to this lifestyle, the only long-term response to rapidly changing environments is an equally rapid adaptation; therefore, plants have developed a very robust signalling and information-processing apparatus. Signalling in plants encompasses chemical and physical communication pathways. Chemical communication is based either on vesicular trafficking pathways, as accomplished also across neuronal synapses in brains, or through direct cell–cell communication via plasmodesmata. Moreover, there are numerous signal molecules generated within cell walls and also as diffusible signals, such as NO, reactive oxygen species, jasmonates, and ethylene, which penetrate cells from the exocellular space. On the other hand, physical communication is based on electrical, hydraulic, and mechanical signals. Besides abundant interactions with the environment, plants interact with other communicative systems such as other plants, fungi, nematodes, bacteria, viruses, insects, and predatory animals. All this great variety of interactions and responses can be embraced within the recently introduced field of plant neurobiology.

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3 Charles Darwin and the Plant Root Apex: Closing a Gap in Living Systems Theory as Applied to Plants

Peter W. Barlow

Abstract Charles Darwin was always pleased to exalt plants in the scale of organised beings, drawing particular attention to the sensory properties of their roots. He even went so far as to say that the root tip acts like a plant brain, located within the anterior end of the plant body. What impressed Darwin was the ability of the root to perceive, often simultaneously, multiple vectorial stimuli, and then make a 'decision' about which bending response to follow. According to J.G. Miller's 'living systems theory' (LST), developed mainly for human organisms and human societies, there are similar sets of 20 subsystems supporting each level of organisation, from cellular to organismic. If LST is a universal theory, it should apply to plant organisms also. About half of all the LST subsystems concern the processing of information. In the present plant-neurobiological context, the information-processing subsystem of particular interest is 'channel and net'. In the light of recent discoveries from plant cell biology, earlier designations of structures to this subsystem are confirmed. They reinforce the idea that plants possess a form of nervous system – even though Darwin denied this particular proposition – which, moreover, makes use of molecules and organelles similar to those found in the neurotransmission systems of animals. The LST approach to plant life converges upon that already recognised for animals and, hence, provides a coherent conceptualisation for the structuring of the two major kingdoms of plants and animals.

3.1 Introduction

In 1880, Charles Darwin, assisted by his son Francis, published their book *The power of movements in plants*. In the last pages of the final chapter the Darwins reflected on the sensitiveness of the tip of the radicle: "it is hardly an exaggeration to say that the tip ... acts like the brain of one of the lower animals; the brain being seated within the anterior end of the body, receiving impressions from the sense organs, and directing the several movements" (Darwin 1880, p. 573). Charles Darwin was much taken with the properties of the root tip. In his 'Autobiography' he records that he "felt an especial pleasure in showing how many and what admirably well adapted movements the tip of the root possesses" (Darwin 1888a, p. 98).

The idea of a plant 'brain' surfaces again in a letter of 1880 from Darwin to Sir Joseph Hooker in which he (Darwin) draws attention not only to his new book but to the root tip in particular: "The case, however, of radicles bending after exposure for an hour to geotropism, with their tips (or brains) cut off is, I think, worth your reading ...; it astounded me" (Darwin 1888b, p. 334).

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The stimuli presented to root tips by Charles Darwin and his son, and to which the roots responded, included gravity, light, moisture, and touch. The Darwins also noticed that two or more simultaneously applied stimuli could be distinguished by a root tip, and that its response was such as to suggest that it could discriminate between the stimuli and judge which was the more important response for the survival of the whole plant. A discrimination between touch and gravity stimuli has been confirmed in recent times by Massa and Gilroy (2003), and between gravity and a moisture gradient by Takahashi et al. (2003).

Whether or not Charles Darwin considered the root-tip 'brain' as a serious postulate, or simply as a fanciful notion, is not entirely clear. The strong advocacy of a root-brain in the mentioned letter to Hooker suggests that he did take this proposition seriously. However, despite the two mentions of a 'brain' and the acknowledgment that a stimulus perceived in one site, such as the root tip, results in an "influence from the excited part" moving to another part where a response takes place, the Darwins disclaimed the possibility of a nervous system: "Yet plants do not of course possess nerves or a central nervous system" (Darwin 1880, p. 572). It may be that the authors refrained from making such a radical assertion because they did not know of any supporting anatomical evidence. In any case, some years were to elapse before convincing images of the neurons of the brain and the central nervous system of mammals were published by Santiago Ramón y Cajal (1909).

Why, then, did Charles Darwin use the term brain? In the absence of any corroboration from anatomical evidence, the possibility of there being a brain was presumably suggested by the evident perception of ambient environmental stimuli by the root tip, the discrimination between stimuli, and the subsequent growth response of the root. A further question is what Darwin had in mind when he wrote that the brain was "seated within the anterior end of the body"? It is not entirely clear whether Darwin was here referring to the brain in relation to the body of a lower animal, to animals in general, or to the body of a plant. And if to a plant, is the 'body' that of the root or of the whole plant? Taking one extreme of all these possibilities, it seems that Darwin viewed the plant as possessing an anterior, or front end. For him, the anterior end was represented by the root tips. Further, in each of these tips there was a brain sensing the environment around the tip and then bringing about a response to those stimuli which the tip was capable of perceiving. The plant brain thereby guided the forward progress of the plant. It mattered not that the shoots failed to follow the movements of the roots: in fact, the shoots mostly remained anchored where they were – except if the plants happened to be geophytes, in which case the shoots would be pulled along by their roots (Galil 1980). The job of the shoots is to engage in sexual or vegetative reproduction (or both) and to scatter progeny.

3.2 The Advancing Root Front and Brain System

By entertaining the notion of an anterior root a rather different perspective of the plant is reached from that which is usually considered. Here, the roots are all important. Their tips form a multiheaded advancing front. The complete set of tips endows the plant with a collective brain, diffused over a large area, gathering, as the root system grows and develops, information important for plant nutrition and survival. Roots also seem to confer a sense of ‘self’ upon the plant (Falik et al. 2003).

The advancing front of root tips, each tip with its brain and sensory surface, can be extensive. For example, after 2 weeks of growth a sorghum (*Sorghum bicolor*) plant may already have developed 2.6×10^3 root tips (Iijima and Kono 1991). If these tips are assumed to be contained within a conical soil volume, the average root density would be 24 tips per cubic centimetre of soil. This value, however, is extremely modest compared with the value of 1.1×10^3 root tips per cubic centimetre which was recorded in the floor of a mixed hardwood forest by Lyford (1974). Tree roots can have up to seven or more orders of branching, tips of each order having their own characteristic dimensions, anatomy, and tropic responses. High-order root branches are, however, often ephemeral and associated with mycorrhizae, a feature which might remove the previously mentioned ‘sense of self’ that normally regulates root development by rendering the root brainless, hence permitting these high root densities.

3.3 The Location of the Plant Root-Brain

3.3.1 Clues from the Transition Zone

Can the location of the ‘brain’ which Darwin considered to reside in the root tip be more clearly defined? To do so would first require agreement upon the definition of a simple brain, such as it being a group of cells that not only receives neuronal-type signals from sensory cells or organs but which also processes those signals, thereby bringing about a response. The response might be recognised as a directional root movement, or tropism.

At this point it is important to distinguish root tropism from root nutation, another type of movement. In fact, a major theme of the Darwins’ book *The power of movement of plants* was that nutation is an inherent autonomous feature of the root tip, and that it is upon this mode of growth

that a tropism is superimposed whenever a new direction of growth is induced in response to a suitable stimulus (Darwin 1880). Much later, the claim was made that the unidirectional movement of root gravitropism actually suppressed the approximately circular movement of the nutation (Ney and Pilet 1981). The two types of root movement – tropism and nutation – might be likened to those animal movements which are responses to the properties of the somatic and autonomous nervous systems, respectively. The animal movements due to the latter system are of innate origin: they are a property of the system itself and are not induced by any external stimulus. Plant nutations are also innate. They are a consequence of an innate program of rotating cell divisions in the meristem which is a function of its cellular structure, whereas, as mentioned, plant tropisms are the consequences of sensory perception and are quite distinct from nutation. Moreover, tropisms are always directional with respect to the vector of the initiating simulus.

There is one site along the root that gathers information for root movements. It is located at the boundary between the root meristem and the subjacent zone of rapid cell elongation, and has become known as the 'transition zone' (Baluška et al. 1996). The modulation of cellular growth within the transition zone may be expressed either one-, two-, or three-dimensionally. In the first case, the transition zone can accumulate cells in linear files and store them in an unexpanded state until some further stimulus triggers their release and subsequent entry into the zone of rapid cell elongation. In the second case of a two-dimensional response, opposite flanks of the root's transition zone show differential growth. Elongation growth is either accelerated or delayed in response to the asymmetric signals received from the root cap, some of these signals being in the form of differential amounts of auxin. The resulting asymmetric, or directional, growth response is registered as a tropism. When the third dimension, which corresponds to the tangential plane, is brought into play, the differential growth of root nutation is the result.

Just as in animals where the brain directly affects the motion of the organism, the cells of the transition zone steer the tropic growth of the plant root in a direction determined by the degree of asymmetry of the incoming signal. In this way, root tips not only avoid potentially hostile regions in the soil but also reach into environments more favourable for the sustenance of the plant.

It seems that, although the transition zone receives asymmetric signals which have their origin in environmental asymmetries, the response of this zone is more akin to that which initiates, in animals, a muscular response under the direction of the coordinating properties of a brain. The transition zone may therefore correspond to a boundary between the body of the root and the efferent portion of the root-brain. Other areas of the root-brain may

exist elsewhere, such as the channels whereby information passes into and out of the root tip. As indicated, a major sensor of information is located in and around the root cap. This is the afferent limit of the root-brain, just as, for example, the eye is the afferent, information-gathering outpost of the brain of a vertebrate animal. Decisions may also be made in this portion of the plant brain as to which tropic response to follow (Takahashi et al. 2003). These so-called decisions might result from conflicts between the respective perception systems.

3.3.2 Clues from the Polarity of Auxin Flow

Besides their sensitivity to external stimuli of various kinds, the perception of which in many cases resides in the root cap, root tips are important sinks for trophic substances which drive their growth. In fact, the evolutionary development of roots themselves may have been a response to a need of the shoots of early photosynthetic plants to rid themselves of excess sucrose by sinking it into new carbonaceous structures which could conveniently be placed underground where their mass would help stabilise the upward-growing shoot. With the development of leaves came not only additional carbohydrate but also the synthesis of auxin (indole acetic acid, IAA), a phytohormone (Sztein et al. 2000). This substance, with its ability to stimulate cell division, may also have played an important role in the evolution of the root. The *chi-chi* on the trunks and branches of old *Ginkgo biloba* trees (Fujii 1895) may, for example, be extant prototypical root organs.

In present-day plants, auxin is transported into the root, and it is here that surplus auxin is catabolised. The growing root tip is thus a sink not only for surplus carbohydrates, but for surplus auxin also. This directed auxin pathway may be instrumental in organising the regular pattern of transverse divisions along the meristematic cell files (Barlow et al. 2004). In the *chi-chi* of *Ginkgo*, by contrast, cell divisions in their tips are oriented irregularly (Kurczyńska and Barlow 1999), possibly as the result of excessive retention of auxin which is only slowly broken down within this organ.

The site within the root where auxin catabolism occurs is the quiescent centre, a small group of a few hundred cells sandwiched between root cap and root proper. The catabolic agent is auxin oxidase, an enzyme whose activity is up-regulated by the incoming IAA (Kerk et al. 2000). The pathway for auxin movement into the root tip is via the xylem parenchyma of the primary tissue. If the plant is secondarily thickened, the auxin moves through the vascular cambium (Schrader et al. 2003). The auxin transport process is mediated by PIN and AUX proteins which, respectively, assist

the auxin flow into and out of the conducting cells (Friml and Palme 2002). The columns of cells which comprise cylindrical plant organs (such as root and shoot) are polarised so that auxin input occurs at the basal end of the cells and auxin output occurs at the apical end.

Some of the auxin which is transported acropetally towards the tip avoids destruction within the quiescent centre and escapes into the root cap. Once in the cap, the auxin becomes linked with the graviresponse system (Ottenschläger et al. 2003) and perhaps with other environment-sensing systems also. Observations from *Arabidopsis thaliana* show that cells of the root cap meristem and the central statenchyme, upon receiving an appropriate gravitational stimulus, can relocate their PIN IAA-exporter protein, AtPIN3 (Friml et al. 2002). Together with AUX1 (Swarup et al. 2001), the gravitationally reoriented PIN3 enables an asymmetric translocation of auxin to take place back along the root. The basipetal movement is under the regulation of PIN2 (Müller et al. 1998) and occurs through root epidermal and outer cortical tissues (Rashotte et al. 2000; Ottenschläger et al. 2003). It brings the auxin to the transition zone, where gravitropism is initiated. The acropetal and basipetal auxin flows are carefully partitioned within different tissues (Blancaflor and Masson 2003), the only route between one auxin flow and the other usually being via the root cap tissue. However, occasional crossover between the two auxin channels may account for observations from which it has been concluded that, in addition to the 'strong' role of the cap in gravitropism, the root tip itself can play a 'weak' role in this and other tropic responses (Wolverton et al. 2002).

3.3.3 The Muscular Root-Brain

Evidence is accumulating that the steps in the process of auxin transport share features that characterise chemical synapses which are instrumental in transmitting impulses along animal nerves (Lodish et al. 2000). Auxin itself should be regarded as a plant neurotransmitter substance (Baluška et al. 2003, 2004) and that its transport occurs via chemical synapses (Barlow et al. 2004; Baluška et al. 2005). The rate of auxin transport is therefore also the rate of propagation of a plant nervous chemical impulse.

Since auxin is transported acropetally into the root tip, and into the root cap, in particular, whence it is redistributed and exported back along the epidermal pathway, the portion of root distal to the transition zone, including the root cap, can be considered to be like a 'brain'. It is here that signals from the ambient root environment impinge, are suitably processed or transduced, and are thence exported via a neuronal-type of efferent channel to the 'muscular' proximal portion of the transition zone to effect

a response. In roots, it seems that this response is effected by motor-type cells within the inner cortex portion of the transition zone, and that these are especially important for 'driving' the movements of tropism and root swelling (Baluška et al. 1993).

The action of animal nerve synapses is facilitated by electrical impulses, and it is these which result in rapid muscle responses. In plants, electrical impulses are also recorded in response to a variety of stimuli, including reorientation within a gravity field (Monshausen et al. 1996). However, it is unclear at present how these impulses relate to the much slower growth responses governed by auxin movement across the plant synapses. It may be that such electrical signals play a different role – for example, in facilitating increasingly rapid responses to repeated stimuli. If so, this would suggest a role for these impulses in simple learning and memory processes in plants (Thellier et al. 2000).

3.4 The Anterior Root-Brain

That auxin transport is largely directed away from the shoot apices and the young leaves, which are the sources of auxin, and is instead directed towards the roots stimulates new thinking about plant morphology. In particular, it is necessary to engage with Darwin's idea of the root being an anterior structure (Darwin 1880, p. 572). There are two common perceptions that might be regarded as reasons for assigning as 'anterior' the head-end of an organism (as in an animal organism). The first is that the property of anteriority is generally associated with an organism's forward movement. The second concerns the presence of a brain. These two propositions also apply to plants. First, the anteriority of the root tips accords with them being the location where rapid forward growth occurs. Generally, at any given temperature, the rates of cell division and cell elongation in root tips are faster than those in the shoot tip by an order of magnitude. Secondly, the root tip, as argued before, is apparently the site of the root-brain. A third criterion of anteriority could be related to the direction of afferent nervous impulses. In animals, afferent nervous activity directs impulses away from what are often peripheral sites of sensory perception, leading them towards the central nervous system and thence to the brain (Tortora and Grabowski 1996). Efferent nervous activity then transmits impulses away from the central nervous system to regions of response, such as the muscles. The major direction of flow of the plant neurotransmitter, auxin, is towards the root tip, where it is then redirected basipetally out of the root cap and back along the tip towards the muscular transition zone (Blancaflor and Masson 2003). The acropetal, tipwards flow of auxin is analogous to the

afferent nervous impulse within the somatic nervous system. The redirected basipetal flow of auxin from the brain composed of root tip and its cap-enclosed sensory tissues is analogous to the efferent nervous impulse. In addition, the movement of auxin, either acropetally or basipetally, occurs in much the same way that animal neurotransmitter substances are moved from neuron to neuron by means of chemical synapses (Lodish et al. 2000).

It seems that one consequence of the polarised flow of auxin towards the anterior root tip is that it leads to the regulation of auxin level by auxin catabolism in the quiescent centre. A second consequence is that a polarised flow can be redirected so that when an appropriate stimulus is perceived differential growth can be initiated. In the plant embryo, also, the flow of auxin is polarised towards the root tip (Friml et al. 2003), this being established after an early phase during which auxin transport is subject to maternal influence. In the embryo, therefore, the root tip can also be regarded as the anterior, or front end, of the plant. It is also worth noting that the root tip is often the first organ to emerge from a germinating seed – an anterior property akin to head-first emergence from hatching eggs or during mammalian parturition.

These observations about anteriority upset the usual perception of the plant where, because of its visibility and upward growth, a false pre-eminence is implied to the shoot. Some authors even explicitly regard the shoot as apical (anterior) and the root as basal (posterior) (Friml et al. 2003). But, for the reasons mentioned, the converse may be a more valid conception of the plant, thereby vindicating Darwin's remark about the anterior root.

3.5 Closing a Gap in Living Systems Theory

The presence of a channel in the form of a cellular system which transports auxin either towards (in the xylem parenchyma) or away from (in the outer cortex and epidermis) the 'brain' of the root tip and its cap, together with the redefinition of the root tip as an anterior element of the plant, results in a congruence between plants and animals with respect not only to their morphology and anatomy but also to the way in which sensory information is processed. It follows that the theoretical analysis of living systems, as it applies to plants (Barlow 1999), can now be completed.

Living systems theory was propounded by James Grier Miller, first alone (Miller 1978), then later in collaboration with his wife, Jessie Louise Miller (Miller and Miller 1981, 1995). In this theory, the organism and the social environment within which it lives and which it has helped to create are analysed into 20 subsystems. These subsystems in turn fall into

Table 3.1. Characteristics of subsystems s11–s20 which process information. (After Miller and Miller 1995)

s11. Input transducer. A sensory subsystem which brings markers bearing information into the system, changing them into a form suitable for further transmission within the system
s12. Internal transducer. The sensory subsystem which receives markers bearing information about significant alterations in subsystems or components, and which converts them into a form suitable for transmission
s13. Channel and net. A subsystem composed of a route or a set of interconnected routes over which markers bearing information are transmitted throughout the system
s14. Timer. A subsystem which transmits to the decider (s18) information about time-related states of the environment or components of the system. This information signals to the decider that certain processes should stop, start, or alter in rate, or advance or delay their phase
s15. Decoder. The subsystem which alters information received from s11 and s12 into a private code used internally by the system
s16. Associater. A subsystem which forms enduring associations among items of information and thus enables the first stage of learning
s17. Memory. A subsystem that carries out the second stage of the learning process, storing information for different periods of time and then retrieving it
s18. Decider. An executive subsystem which receives information inputs from all other subsystems and transmits to them information outputs
s19. Encoder. The counterpart of s15 whereby information is converted from a private code used internally by the system to one which can be interpreted by other nearby systems
s20. Output transducer. The subsystem concerned with the output of informational markers into the channels in the systems environment

three main groups concerned with the processing of (1) matter–energy and information, (2) matter–energy alone, and (3) information only. The organism and its supraorganismic environment are also deconstructed into a hierarchy, the first four levels of which are (1) cell, (2) organ, (3) organism, and (4) group. Each level of organisation owes its existence and identity to the same set of 20 subsystems. These subsystems, and the processes which they contain, support and define each level of organisation. It is this last point which accounts for the fact that the levels of organisation show self-similarity. That is, each level is supported by a similar set of subsystems, though, obviously, the items which correspond to each subsystem materially differ at each level. The processes or properties that characterise the third main group of information-processing subsystems are listed in Table 3.1. They are the ones relevant to the plant-brain problem.

In an earlier essay on the relation between living systems theory and plant life (Barlow 1999), it was clear that there were gaps and uncertainties in the

analysis. In particular, it was difficult to discover correspondences between the subsystems which the theory suggested were involved in information processing and any known processes, or plant structures. The question is whether, in the light of new knowledge and the possible existence of

Table 3.2. Subsystems which process information at four levels of organisation that involve plant organisms

Subsystem	Level			
	Cell	Organ	Organism	Group
s11. Input transducer	Plasma membrane sites bearing AUX1 receptor protein	Statoliths	Root cap, sensory hairs	Sensitive plants at the boundary of the group
s12. Internal transducer	Endocytotic vesicles	Statenchyme	←	–
s13. Channel and net	Actin cytoskeleton, plasmodesmata	Plant synapses, pit fields	Supersymplasm	Vegetation patchwork
s14. Timer	Biochemical oscillators, mitotic clock	Cells perceptive of external timers	Canopy and its properties	Autumn colours
s15. Decoder	Release of auxin	←	–	–
s16. Associater	Crosstalk molecules	Cells with inductive properties	Organs that respond to acclimation and aptation	–
s17. Memory	Short-lived gene regulators	Hysteresis loops, transcellular electrical impulses	←	–
s18. Decider	Regulator genes, osmoregulators	Transition zone cells	Collective plant brain, target cells	–
s19. Encoder	Genes and proteins involved in hormone response	Auxin response processes	Organ aposematic marking	Plants with open flowers
S20. Output transducer	Plasma membrane and PIN proteins	Cells which export information to motor cells	Attractor organs, scent glands	Reproductive individual

An *arrow* indicates that the subsystem is devolved to the next-lower organisational level

a plant nervous system, the corresponding plant subsystems can be more confidently identified; and if so, whether they are concordant with those analysed by Miller and Miller (1995) for animal/human systems. Table 3.2 summarises the present position for the various levels of plant organisation.

Of the information-processing subsystems listed in Table 3.1, s14, timer, is not directly relevant in the present context as it deals with the timing of processes in relation to innate or external cues. At the levels of the cell and organ, subsystems s16 and s17, associater and memory, respectively, may have correspondences with the fast electrical signals that can be propagated along plant organs. The remaining subsystems are those which either transmit information in the form of an auxin flow (especially s13, channel and net, at the organ and organism levels), or are concerned with the transduction of external information into the body of the plant and its conversion into internal information for eventual transduction as an output response.

In whatever form information is perceived by the plant, it is internalised and processed in such a way that it can be ultimately directed towards movement or reproduction. The movement of plant organs involves tropisms (nastic movements are excluded for the moment owing to their autonomous nature), the informational inputs for which are mainly gravity, illuminance and moisture. Auxin is the key mediator of these growth events.

Although plant life can be decomposed into a series of hierarchical levels, these are more than merely classificatory conveniences but are systems of dynamic interactions. Thus, the states of the lower organisational levels provide conditions for the emergence of larger-scale organisations such as the group or community, and in particular for the physical movements of the latter whereby they – individuals, groups, communities – are able to occupy new environments by means of propagules adapted and dispersed for this purpose (seeds, abscinded plant parts, etc.). Finally, the communal movement of plants aids in the construction of a green energy receptor and transducer – and maybe something more (Miller and Miller 1982) – which covers the surface of the Earth. In some unfathomable way, this living skin may even fulfil the role of a subsystem within some much higher level of organisation and thereby participates in the ongoing creative evolution of organic living systems.

3.6 Conclusions and Future Prospects

Was Charles Darwin prescient in his use of the word brain in connection with his studies of the activities of the plant root apex and the implications that might flow therefrom? We shall never know. Given his unease over the

reception of his book *The origin of species*, he might well have kept any ideas about plants possessing brains strictly to himself.

One attribute of a brain, as the term is commonly understood, is that it is an organ with a definite structure and location which gathers or collects information, which was originally in the form of vibrations (heat, light, sound, chemical, mechanical, ...) in the ambient environment and somehow transforms them into an output or response. Interestingly, the execution of these responses is by means of another vibrating system – the circumnutating root (Darwin 1880). Any change in the direction of the stimulus temporarily overrides the nutational process until the usual orientation of the plant organ is regained. Maybe this was how Darwin understood the situation. However, the absence of obvious nervous tissue would have been a serious problem in his further pursuit of the matter. But the situation has changed in recent years, as other chapters in this book will show. With the discovery within plants of many of the components of animal-type nervous systems (Baluška et al. 2004), the question is how this new knowledge can be conceptualised, and whether any paradigm will emerge which acknowledges some sort of plant nervous system; and moreover, whether this nervous system will have features which can be generalised to all eukaryotic organisms.

In recognising the possession, by the root apex, of a brain-like function, a lacuna is filled in applying Miller's comprehensive living systems theory to plants. Consequently, there is now scope for analysing the information-processing subsystems and linking them with the other two major types of subsystems that process matter and energy (Barlow 1999). This task can be done at the various levels of organisation that characterise all biological constructions.

The subsystems are not static artefacts of theory, but represent inherently dynamic processes by which biological constructions act. They apply not only to large constructions such as social communities, but also to smaller microcosms such as cells. Indeed, the subsystems fulfil the role of the 'coordinative conditions' which the physicist and systems analyst Lancelot Law Whyte had seen as providing a key to understanding biology in general: "Until the coordinating conditions have been identified no theory of phylogenesis, of ontogenesis, or of their relations, can be regarded as definitive. Moreover [they] hold the clue to the relation of physical laws and to the unity of the organism" (Whyte 1965, p. 67). For Whyte, these conditions express "the biological spatio-temporal coordination, the rules of ordering which must be satisfied ... by the internal parts and processes of any organism capable of developing and surviving in some environment. The coordinating conditions are the expression of geometrical, 3D, or perhaps kinematic rules determining the necessary 3D or spatio-temporal network of the atoms, ions, molecules, organelles, etc. in a viable organism. They ...

must cover all the fundamental aspects of the unity of organisms" (Whyte 1965, p. 6). The recognition, as well as the appreciation of the place of these conditions – or subsystems, as they would be in Miller's living systems theory – within the hierarchical construction that comprises plant life on Earth, provides a realistic and holistic way of perceiving and responding to what our senses tell us, both directly and intuitively, about plant life.

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4 How Can Plants Choose the Most Promising Organs?

T. Sachs

Abstract Branch death is an important component of the processes that generate tree form. Many branches are formed and the plant appears to "choose" the most promising alternatives for survival and further development. The purpose of this chapter is to consider how this choice could occur in the absence of a central decision center. Experiments were carried out on a model system of pea seedlings with two shoots. In most plants only one of these shoots continued to grow. We studied the conditions that influence the choice of the "winning" branch. These included removing entire branches or leaves at different ages and modifying the environment of the entire plant or of individual shoots. The evidence from experiments and from comparative observations of many trees supports the following hypothesis. All components of a shoot are sources of auxin and possibly of other signals. The level of auxin synthesis depends on the immediate environment and the developmental stage of individual leaves. The responses to auxin include the orientation of vascular differentiation towards organs that are its strongest source. This oriented auxin response results in competition between alternative organs. This is a prominent example of "developmental selection," an alternative to developmental programs and prepatterns, which also has various other roles in the generation of biological form.

4.1 Introduction: Developmental Selection of Branch Configurations

Plants develop their complicated forms by reiterating developmental processes, forming repeated structural metamers (Halle et al. 1978; Barlow 1989). This can be readily verified by following the development of the young tips of leading branches. Reiteration is also necessary on theoretical grounds, for there is no other way in which the information coded in a genome could suffice to determine the detailed form of a large tree. Yet reiteration cannot be the whole story. Even a superficial observation of most trees suggests that while all buds are potential branches, most of them do not develop further. Those that do become branches develop at varied rates that depend on their immediate environment and the presence of neighboring branches, not only on a reiterated program. Further, the majority of the branches that start growing in a young canopy are shed within a few years (Sachs and Novoplansky 1995). Dead branches are readily found below large trees, especially following a storm. The shedding of branches is also the origin of bare trunks, which commonly develop from

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branched systems, similar to those seen in the upper parts of the very same trees. Reiterated developmental processes are thus only a framework that is subject to long-term modification.

The purpose here is to consider the mechanisms that determine which branches are retained. This question calls for careful, repeated experiments in defined conditions, for which trees are hardly suitable. The work described here is therefore based on a model system, using seedlings of an annual plant. The results suggest that branches compete, and possible mechanisms of this internal competition are briefly considered. The discussion proposes that competition and selection which follows rather than precedes initial development is common at all levels of biological organization.

4.2 An Experimental Model Demonstrates Branch Competition

4.2.1 The Experimental System

The development of annual plants has been used extensively for studies of the inhibitory influence of a growing shoot on bud growth (Snow 1937; Sachs 1991; Cline 1994). The choice of a successful branch from among a number that have started growing does not, however, usually occur in annual plants. A possible evolutionary reason is that branch shedding is not adaptive in plants with a short life span (Novoplansky et al. 1994). A valuable model system has nonetheless been developed in annuals (Snow 1931; Sachs 1966; Novoplansky et al. 1989). It uses the unstable state of a plant regenerating after severe damage. The first or seminal shoot was removed from pea seedlings (Fig. 4.1a). This treatment distorted the balance between the shoot and root systems of the growing plant, and, as in cut trees, shoot number was increased (Sachs et al. 1993; Sachs 2004). Two buds grew into more or less equal shoots or branches (Fig. 4.1b,c). Gradually these two shoots became increasingly unequal (Fig. 4.1d). The smaller shoot stopped growing and in some varieties it eventually died, returning the plant to a single axis state (Fig. 4.1e; Sachs 1966). The deterioration of the smaller shoot was prevented whenever the larger shoot was removed (Fig. 4.1f), demonstrating that interactions between the shoots are a major determinant of their individual fate.

“Two-shoot” pea seedling are an experimental system in which it is possible to ask how the plant chooses the shoot that continues developing. The following simple experiments demonstrate that there is no key parameter. Instead, the plants appear to be integrating information from varied sources.

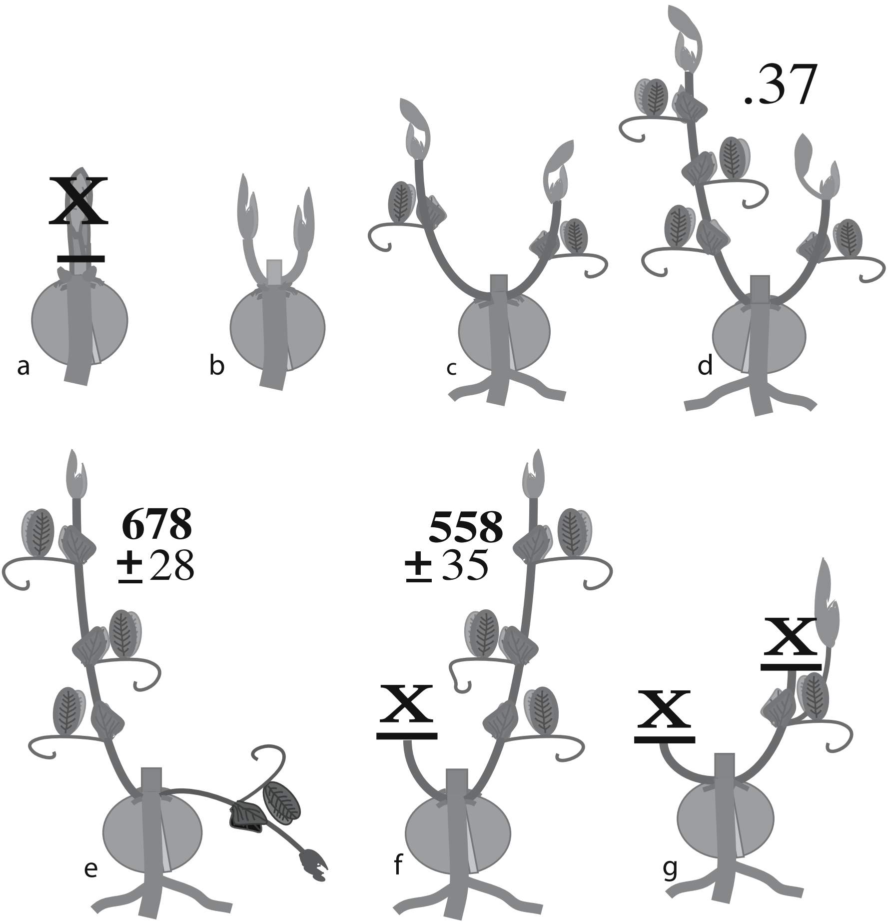


Fig. 4.1. The two-shoot pea experimental system. **a** The seminal shoot of a germinating pea seedling was removed. **b** Within 3 days the removed shoot was replaced by the growth of inhibited buds and they became two new shoots. **c** Within four additional days the new shoots carried expanded leaves and were generally somewhat unequal. **d** Inequality between the shoots increased. At the time the experiments shown in Fig. 4.2 were started the wet-weight ratio of the smaller over the larger shoot was 0.37. **e** In some varieties the smaller shoot actually died within 2 weeks. **f** Removal of the larger, dominant shoot before the smaller shoot had died. The declining shoot survived and developed rapidly. Its average fresh weight, shown in milligrams, did not differ much from that of an undisturbed dominant shoot (in **e**). **g** Dominance influences between shoots. Removing both the dominant shoot and the upper part of the smaller shoot resulted in the growth of a bud in the axil of an expanded leaf. This bud did not grow when the large shoot was left intact. (Based on Sachs and Hassidim 1996)

4.2.2 Stress Increases Competition

The two-shoot pea plants were stressed by removing parts of the reserve seed tissues (the cotyledons, Fig. 4.2b). This treatment, in addition to the obvious reduction in shoot growth, also increased shoot inequality (Sachs and Novoplansky 1997). When the larger shoot was removed the weaker one always survived and grew to regenerate a new plant (Fig. 4.1f). This suggests that the weaker shoot is responding not only to stress but also to an enhanced competition with the stronger shoot. Such results were not limited to cases where there was a reduction of reserve storage materials. The stress of weak light and limited mineral nutrition had the same effects (Fig. 4.2c,d).

4.2.3 Unequal Light Conditions

The two shoots could be subjected to different conditions, simulating environmental heterogeneity at the level of an individual plant (Novoplansky et al. 1989). Reducing the light available to one of the two shoots strongly inhibited its growth and increased shoot inequality (Fig. 4.3e). When light was excluded from one shoot, it died within 2 weeks in 80% of cases (Fig. 4.3f). Here, again, the response depended on the presence of the shoot that remained in the light. When the more fortunate shoot was removed, the darkened shoot elongated rapidly, forming small, yellow leaves. This is the common form of seedlings whose entire shoot system is in dark conditions, part of the etiolation syndrome.

4.2.4 The Rate of Shoot Development and Leaf Removal

The inequality of the two shoots tended to increase with time, and relations were not reversed unless the individual shoots were subjected to additional treatments. This suggests that relative shoot size had an influence on the subsequent development. Size is a parameter that can be readily manipulated, simulating natural herbivory. When the entire top part of one shoot was removed the shoot could continue development only by means of one or more of the buds subtended by its remaining leaves. Such development occurred readily, but only when the competing intact shoot was removed (Fig. 4.1g). A bud is thus at a pronounced disadvantage relative to a growing shoot apex. This is, of course, the bud inhibition mentioned before, the

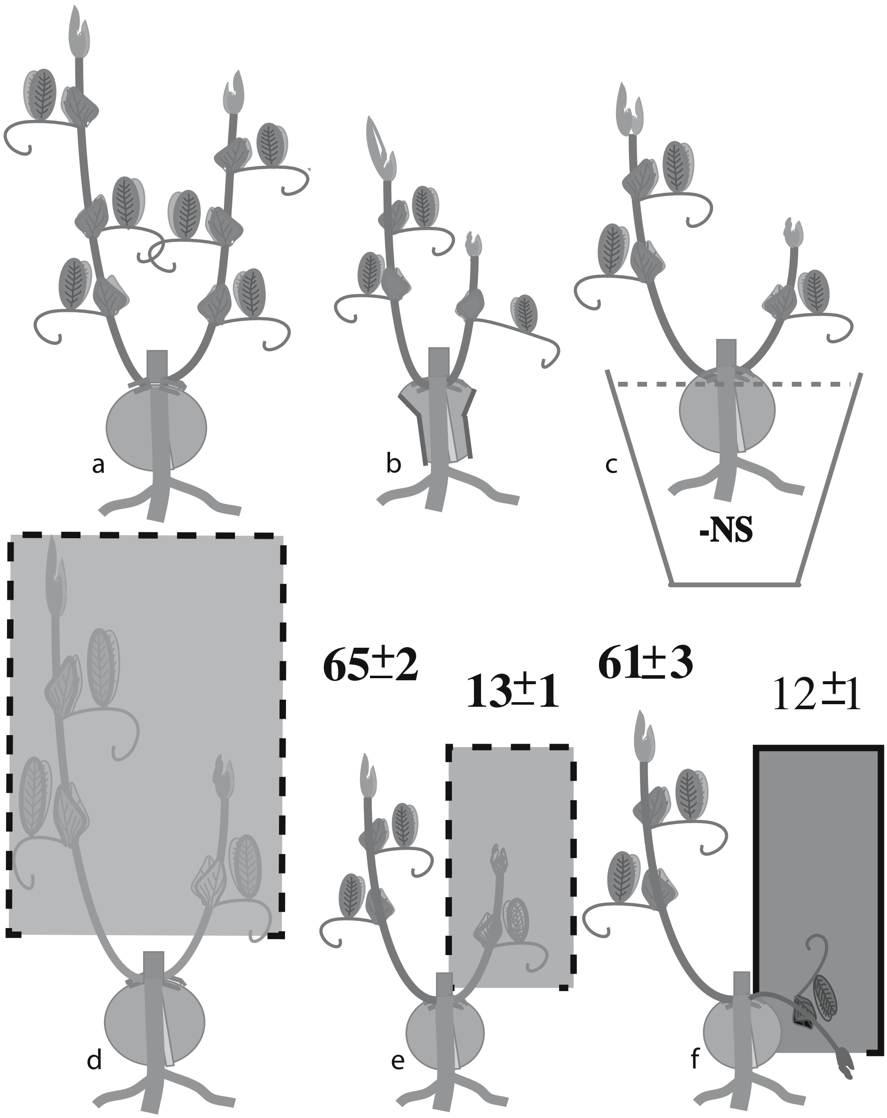
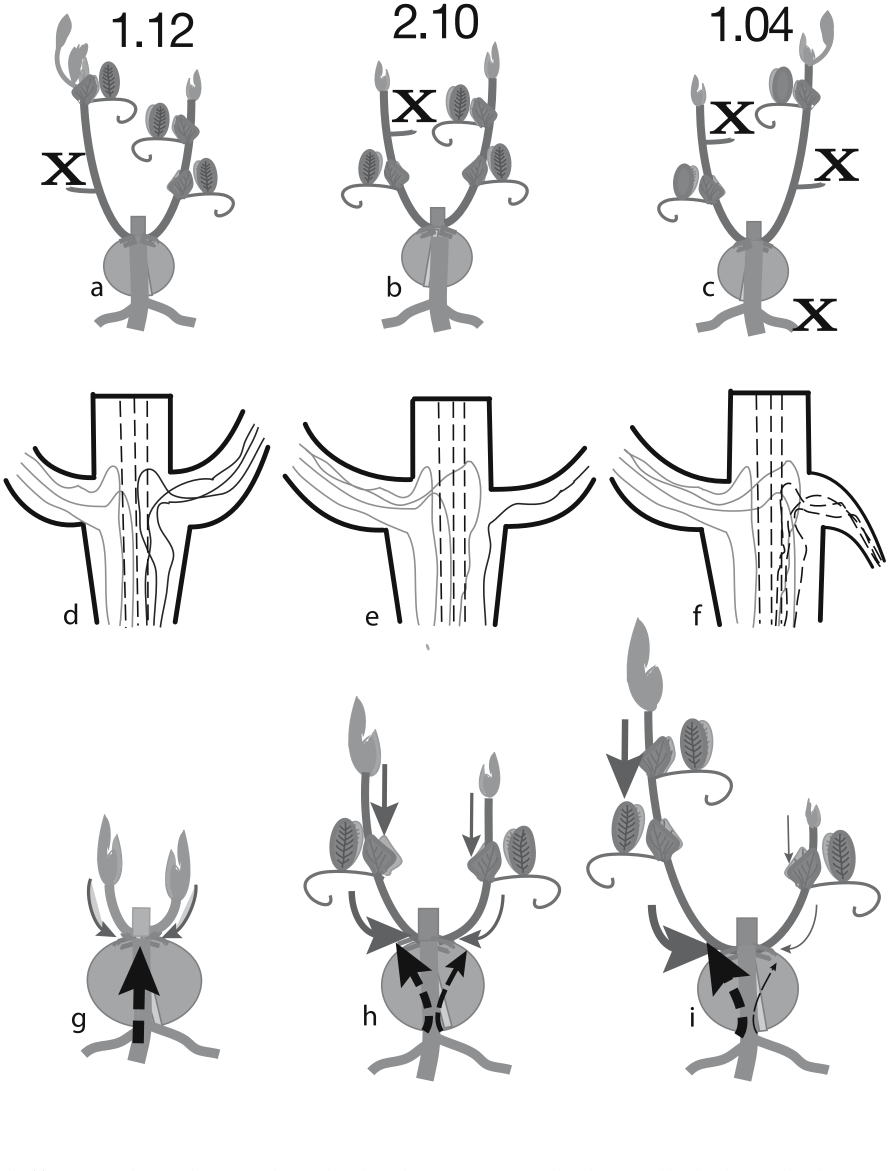


Fig. 4.2. Responses of two-shoot peas to overall and to localized stress conditions. **a** Both shoots developed in most control plants in optimal conditions. **b** Removing a large part of the cotyledons, and hence of the reserve materials, increased shoot inequality. **c** Growing plants in vermiculite without nutrient solutions (*NS*) increased shoot inequality. **d** Shading both shoots, which presumably limited photosynthesis, is a third example of the general increase of shoot inequality in response to stress. **e, f** Restricting the light reaching only one of two young shoots greatly limited its development. When a shoot was darkened (**f**) rather then shaded (**e**) it died in 80% of the plants. The *numbers* refer to the dry weights of the shoots in milligrams. (They are not comparable to the fresh weights in Fig. 4.1.); (Based on Sachs and Novoplansky 1997 and Novoplansky et al. 1989)



difference here being that the bud is not morphologically below the leaves that inhibit its development. Space limitations preclude the consideration of quantitative evidence that developmental rates are of critical importance in the criteria used by the plant (Novoplansky 1996, 2003).

Finally, individual leaves can be removed (Sachs and Hassidim 1996). When this was done to the larger of two somewhat unequal shoots there was an increased chance of its losing a dominant position (Fig. 4.3a, cf. Fig. 4.1d). The removal of a developing leaf has a larger effect than the

◀ **Fig. 4.3.** **a–c** The influence of simulated herbivory on shoot relations. The *numbers* are the ratios of the fresh weights of the initially smaller over the initially larger shoot after adding the expected weight of removed leaves; they should be compared with the control of 0.37 (Fig. 4.1d). **a** Removing a mature leaf at an early stage from the somewhat larger shoot resulted in the two shoots being more or less equal. **b** Removing a young, expanding leaf from the larger shoot had a larger effect than removing a mature, photosynthetic leaf. **c** An expanding leaf was removed from the larger shoot and a mature leaf from the smaller shoot. The two shoots grew to be essentially equal, dramatically changing the outcome seen in the controls. **d–f** The evidence of vascular differentiation. The *lines* are only indications of overall structure, not a record of all the numerous vascular channels. **d** The cambial space that had originally been occupied by vascular channels (*broken lines*) that led to the removed seminal shoot (Fig. 1a) was taken over by new connections of the new growing shoots with the roots. There were no direct contacts between the new shoots. **e** Where the two shoots were unequal their vascular contacts and the space they occupied were correlated with their size and developmental rates. **f** When a weak shoot died the space occupied by its vascular channels, but not the channels themselves, was taken over by the growing shoot. **g–i**. An hypothesis: the choice between shoots is mediated by hormonal relations with the roots. The shoots are the source of signals that inform the plant about their state. The response to these signals that they receive from the roots limits their development. **g** In the cut plant essential supplies from the root are present in excess, and the shoots do not compete. **h** As the shoots develop, their young and, to a lesser extent, their mature leaves increase their signals and their requirements. A large proportion of the supplies from the roots are diverted towards the larger shoot and they enhance its continued development. **i** A positive feedback between development and continued development ensues and an increasing proportion of the resources are diverted towards only one of the two shoots. (Based on Sachs and Novoplansky 1997)

removal of a photosynthetically active leaf (Fig. 4.3b). A plant forced to "choose" between two damaged branches discriminated against the one that had lost a young rather than a mature leaf (Fig. 4.3c).

4.2.5 Hypothesis: Branches Compete

The results of the experiments with pea seedlings suggest that branches of the same plant compete for resources, their genetic identity not withstanding (Sachs et al. 1993; Sachs 2004). This competition is temporarily lifted when the shoot/root balance is disrupted by the removal of the seminal shoot and is intensified when the plants are in stress conditions. Competition means that the plants not only respond to local environmental conditions, such as low light and local leaf damage, but they also actually "choose" for continued development the most promising of the available alternatives. An important criterion for this choice is rapid development, which predicts future rather than immediate photosynthetic performance.

4.3 Mechanisms of Competition

The statement that branches of the very same plant compete calls for concrete mechanisms. These should allow for a comparison of branches and for the selection of the more promising alternative. Such comparisons must occur in the absence of a central "brain" or computing center, an organ which plants lack. Possible alternatives must take into account the fact that plants can have numerous branches, and it is impossible for each to be the source of a unique signal. Since competition is influenced by resource availability, an obvious possibility is that the stronger branches act as sinks that divert transport towards themselves (Henriksson 2001). This is supported by evidence that the vascular channels passively transport materials to the sinks in which they are consumed. Yet both young and mature leaves, which have opposite sink effects, act to enhance the role of a strong branch (Fig. 4.3a–c). A more general reason that sink effects could not suffice is that branch competition is a long-term process, during which new vascular channels differentiate (Sachs et al. 1993). Vascular differentiation, unlike short-term transport, is actively oriented so that it connects the dominant branches with the rest of the plant (Sachs 1991; Berleth and Sachs 2001). Since hormones and essential substrates move along the vascular tissues, their long-term transport is in fact oriented towards dominant branches.

When considered in terms of vascular differentiation it is easy to observe the "conflict" between the vascular connections of branches that develop on opposite sides of the same axis (Fig. 4.3d–f). There are no direct vascular contacts between branches: all channels within the plant axis are polar, connecting shoot and root tissues (Sachs 1991). The larger the branch the larger its vascular supply and the more axial space this supply occupies. The causal relations between the branch development and oriented vascular differentiation can be readily confirmed by branch removal. The molecular basis for processes of reoriented vascular differentiation could be dependent on changes in the localization of the products of PIN genes (Palme and Gälweiler 1999). The suggested role of vascular differentiation focuses competition on the orientation and activity of cambial cells where developing vascular systems meet (Sachs et al. 1993). This is not a proof, however, since the evidence does not show that orientation precedes rather than follows branch development.

The hypothesis can be taken one step further (Fig. 4.3g–i). Leaves are known to be sources of the hormone auxin (Sachs 1991; Berleth and Sachs 2001; Ljung et al. 2001). This same auxin induces the differentiation of new vascular tissues along the axis connecting its source with the roots (Sachs 1991; Berleth and Sachs 2001). Auxin is the only known signal whose local source actually determines the orientation of these vascular tissues. The

formation of auxin occurs in all parts of a developing shoot, but especially in young expanding leaves (Ljung et al. 2001). Though the quantitative data are meager, available knowledge about auxin synthesis and vascular differentiation suggest that its synthesis is enhanced by light. Since neighboring branches shade one another, competition is environmental, not only internal (Sachs et al. 1993). Yet if shade influences auxin formation both types of competition could reflect the same internal mechanism by means of competitive vascular orientation. All this is in accordance with the hypothesis that auxin is a signal that integrates the information about the location, size, environment and rate of development of a growing branch (Sachs 2004).

Are there other possible mechanisms of branch competition, in addition to auxin-induced orientation of vascular differentiation? These mechanisms need not be mutually exclusive and it is possible, or even likely, that more than one has a role in a process as central as the relations between plant organs. An alternative to oriented differentiation is the adaptation of plant tissues to the higher level of auxin that is supplied by the stronger branch (or other signals that a shoot may produce). The cambium thus responds to the "best" branches – the ones that are the strongest sources of auxin – and "ignores" the weaker branches, whose vascular tissues deteriorate without being replaced. This possibility is plausible and it would account for evidence that interactions between branches need not require vascular reorientation (Snow 1937). However, there appears to be no concrete evidence about its cellular basis and the way adaptation to high auxin could spread through plant tissues.

4.4 Conclusions and Future Prospects

A general conclusion goes beyond the relations between tree branches. Similar processes of developmental selection could have a large role in biological pattern formation (Edelman 1987; Sachs 1988, 1991, 2002; Frank 1996, 1997). This selection actually generates information about the location of the different structures, such as branches, during development itself. In this it differs from programs or prepatterns in which detailed information precedes actual differentiation. Developmental selection shares principles with the Darwinian mechanism of evolution (Frank 1997), though there is an important difference. The various branches of a tree are genetically identical and the outcome of their selection is an adaptive form, not an evolution of a new genetic system. Conflicts of interest between alternative branches or other structures of the same organism do not arise.

The specification of form by stochastic variation followed by selection appears to be counterintuitive, but it is supported by direct observations of

the development of varied biological patterns. The mature stomata of *Sansevieria* are more orderly or predictably spaced than the early, reversible stages of stomata initiation (Kagan and Sachs 1991). "Neural Darwinism" is a mechanism by which appropriate nerve connections are selected from excess alternatives (Edelman 1987). Within cells, microtubules are maintained according to functional roles that are established after they have been formed (Kirschner and Mitchison 1986).

It may be useful to consider the adaptive significance of the generation of tree form by developmental selection rather than strict programs. Competition between branches requires the wasteful development of structures that are not maintained. There is no doubt that dead branches below trees represent considerable organic material. Yet these branches did carry out photosynthesis for as long as they were productive and resources such as bound nitrogen can be withdrawn from dying plant organs (Habib et al. 1993). Losses of substrates could be balanced by the advantages of gradual selection, in which information about the value of an existing branch feeds back to its survival and further development. This testing of varied possibilities could provide for a robust outcome.

A major advantage of developmental section may be in its relation to plasticity. It is possible that in a predictable, ideal world strict programs could result in superior biological forms. The real world, however, is far from predictable. In the case of trees, the presence of competing neighbors, damage due to herbivores and parasites and possible local failures in the development of the plant itself require a pronounced developmental plasticity. Developmental selection uses the very same processes and genetic information to generate form and to insure plastic responses to unpredictable conditions (Sachs 2002). This parsimony might have adaptive significance both because simpler developmental systems are required and because the various component are all optimally integrated.

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5 The Role of Root Apices in Shoot Growth Regulation: Support for Neurobiology at the Whole Plant Level?

Peter M. Neumann

Abstract This chapter reviews the potential and actual role of plant neurobiological activity for integrating function at the whole plant level. Some support is provided for the drawing of analogies between the signaling roles of plant long-distance transport systems and animal nervous systems. However, the specific findings reviewed here do not indicate that root apices function as essential neurobiological command centers involved in regulating shoot growth responses to adverse changes in the root environment.

5.1 Introduction

How cell and tissue activities give rise to integrated function at the level of the whole organism is a central question in biology. Hormonal signal molecules and the neurobiological activities of interconnected nervous systems and brains can provide the needed integrative capacity in animals. In plants, the activities of hormonelike substances are well characterized but neither morphologically distinct brains nor anatomically distinct nerve cells appear to have evolved. Nevertheless, it has been suggested by Baluska et al. (2004a, b) that the transition zone in the growing root tip [which also shows intense genetic (Birnbaum et al. 2003; Bassani et al. 2004), hormonal (Aloni et al. 2004) and ionic activities (Peters and Felle 1999; Fasanao et al. 2001; Fan and Neumann 2004)] together with the vascular transport tissues (xylem and phloem) that interconnect with practically all the parts of higher plants may be compared with the brains and nervous systems of animals.

A criterion for a good hypothesis is that it stimulates thought and theoretical or experimental tests of validity: The suggestion that the root apex of higher plants is part of a cell body that has neurobiological parallels and can be viewed as a "diffuse plant brain" connected via vascular strands (the "nerves") to the plant shoot certainly meets this criterion.

When I, a plant stress physiologist, first considered the plant neurobiology concept, I was stimulated to clarify for myself the function of nerves and brains in animals and to evaluate the potential need for similar systems in plants. I was also stimulated to look at the evolutionary progression of plant morphology and anatomy in order to better evaluate the essentiality to plant life of roots and vascular tissues serving as neurobiological entities.

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Finally, I examined the plant neurobiology concept in the light of several experiments from our laboratory. These involved the effects of partial root excision, or of treatments which inhibit root activity, on root-to-shoot signaling and shoot growth responses to environmental stresses. The findings are presented in the following three sections.

5.2 The Comparative Need for Rapid Neurobiological Activity in Animals and Plants

Multicellular animals and plants appear to have evolved from a common ancestral cell type and they share many basic molecules together with life-associated activities such as respiration, cell division and long-distance transport systems. However, there are, of course, intrinsic differences between animals and plants. For example, brains and networks of specialized nerve cells have clearly evolved in animals but not in higher plants. The animal brain can be viewed as a device which rapidly processes sensory input signals, makes decisions and sends motor output. The human brain consists of around one hundred billion neurons intimately connected via the spinal chord to a nervous network which can rapidly transmit signals to and from every point in the body. The brain of higher animals is essential to life since decapitation or brain inactivation immediately leads to death of the organism. In contrast, excision of roots or root apices of plants need not have immediate adverse effects on the remaining parts (consider cut flowers and see Sect. 5.3). Centralized brains are, however, not essential for the existence of some types of animals. For example, members of the marine phylum Cnidaria (e.g., hydra, jellyfishes, corals, sea anemones and Portuguese man-of-war) have survived and thrived for about a half billion years without brains. Body control in these organisms is based on a diffuse nerve net which provides the communication between sensory cells and the muscle cells involved in essential movements. Cutting nerves in lower or higher animals is expected to result in the paralysis of associated muscular function and is likely to be life-threatening. In contrast, phloem girdling (i.e., interruption of potentially nervelike tissues) in branches of fruit trees, as practiced by farmers in Israel, does not appear to have much adverse effect and can result in the production of larger and sweeter fruits. Thus, plants and animals can differ in their responses to disruption of signaling events.

A more important and major difference between animals and plants is that the animals need and use contractile muscles to facilitate their heterotrophic life style, while plants do not. A heterotrophic life style means that animals depend on obtaining preformed organic foodstuffs from their environment. Regulated patterns of muscular activity and directional mo-

bility are needed to allow animals to hunt, ingest and digest essential organic foodstuffs and to actively excrete resultant solid or liquid waste products.

In contrast, plants have an autotrophic life style, i.e., while remaining stationary they can synthesize all the life-essential organic materials they need from readily available supplies of carbon dioxide, light, water and mineral nutrients. Moreover, waste products can be stored internally by plant cell vacuoles. On the other hand, plants may produce up to 100,000 secondary compounds needed, for example, as defense against herbivores and diseases, or to help attract pollinators (Poethig 2001; Lev Yadun et al. 2002).

In animals, aural, olfactory, visual and tactile input signals are involved in feeding and in additional essential activities such as prey capture, fighting, fleeing or mating. These varied inputs need to be continuously processed by nervous systems in order to produce the appropriate muscular contractions within seconds. Speedy signal transmission and processing is clearly essential to animal survival. Plants appear to generally operate within a slower time scale than animals. For example, the important and relatively fast stomatal changes induced by soil water deficit or darkness take minutes rather than seconds to complete (Kramer and Boyer 1995). Similarly, significant geotropic responses to changes in root position take at least 10 min to establish (Fasano et al. 2001; Aloni et al. 2004). Finally, stem growth inhibition by a severe soil water deficit in a cactus species took weeks to establish (see later). Of course every rule has its exceptions and rapid mechanosensory responses such as leaf folding in *Mimosa* or trap closure in Venus's-flytraps can take only seconds to complete. The action potentials and chemical stimuli associated with rapid leaf folding in *Mimosa* can certainly be compared with the electrical and chemical processes involved in nerve signal transmission. However, the velocity of propagation of electrical signals in plants is about 2 cm s^{-1}, whereas action potentials travel along nerve cells at tens of meters per second (Salisbury and Ross 1985).

In summary, the immobility of plants and their generally slower responses to environmental changes suggest that a plant capacity for rapid long-distance signal transmission via nervelike cells and rapid signal processing in brainlike centers of activity is less essential than it is in animals.

5.3 Plants That Manage Without Roots, Root Apices and Vascular Tissues

The plant neurobiology concept would be supported if functional essentiality of the root apex "brain" and associated vascular system "nerves" for the continuity of plant life could be established. In this respect it is interesting to trace morphological and anatomical changes which occurred in the

evolutionary progression from lower to higher plants. This progression indicates that lower forms of plant life without differentiated roots or vascular tissues have survived successfully to this day (Raven et al. 1999; Poethig 2001). For example, multicellular brown algae such as the giant kelps found in the Pacific Ocean show plasmodesmatal connections between cells as in higher plants and often consist of a leaflike blade connected to a stemlike stipe. Moreover, some kelps have elongated cells in the center of the stipe which resemble the phloem transport tissue of vascular plants. Some kelps may also develop a basal holdfast structure situated at the base of the stipe. This anchors the algae to rocks and superficially resembles a root. However, other kelps have no holdfast and form free-floating masses. Thus, large marine algae appear to be able to function successfully with or without differentiated roots and vascular tissues.

The first multicellular terrestrial plants may have been similar to the small multicellular green algae *Fritschiella*, which has some common features with present-day higher plants. *Fritschiella* has plasmodesmata and forms multicellular rhizoids at the soil surface from which erect branches sprout (N.B. although rhizoids occupy the same position as roots they have no specialized apices and no differentiated transport cells). *Fritschiella* can therefore function successfully without specialized conductive tissues to facilitate nutrient and signal transmission and without complex root apices.

Sporophytes and gametophytes of bryophyte mosses are thought to represent a subsequent stage in the evolution of higher plants and reveal more similarities. Thus, the cells of bryophytes are interconnected by plasmodesmata and leaflike structures may be born on a stemlike structure which can be attached to rhizoids at the base. Moreover, the stems of some bryophytes may include central strands of nonlignified water-conducting tissues and of nutrient-conducting tissues. Although some bryophyte species utilize primitive conductive tissues, it is again noteworthy that others function successfully as multicellular plant organisms despite the absence of conductive tissues and of root apices.

In summary, various multicellular plant organisms can function without roots, root apices or vascular tissues (Table 5.1). In contrast, modern gymnosperm and angiosperm plants share a body architecture which includes roots, root apices and vascular tissues. The relatively large size and world-wide distribution achieved by gymnosperm and angiosperm plants suggest that they represent a winning formula for ecological success. Nevertheless, many lower forms of plant life have retained an ability to survive and propagate very successfully, in a world-wide variety of different environments, despite the absence of these attributes. The degree to which the relatively greater success of the higher plants is attributable to the facilitation of plant neurobiological activity by root tip “brains” and vascular

Table 5.1. Presence or absence (+ or –) of characteristics which may be associated with neurobiological activity in photosynthetic organisms

	Kelps	Green algae	Mosses	Higher plants
Plasmodesmata	+	+	+	+
Vascular tissue	+/–	–	+/–	+
Hold fast	+	–	–	–
Rhizoid	–	+/–	+	–
Root	–	–	–	+

system "nerves", remains to be determined. An obvious additional explanation for the success of the higher plants is that differentiated root systems and vascular tissues increase plant capacity to effectively access and/or distribute water, mineral nutrients and organic solutes to all parts of the whole plant structure.

5.4 Do Plant Shoot Responses to Environmental Stresses Require Rapid Root-to-Shoot Signaling?

Experiments in which any brainlike activity in the plant root apex or chemical and electrical signal transmissions via vascular tissues are inactivated may reveal the degree to which neurobiological activity is essentially involved in the functioning of the whole plant. In this section, three experiments which examine the effects of partial or complete inactivation of the roots on shoot growth responses to environmental change are reviewed.

In the first experiment we examined growth and physical characteristics of the emerging first leaf of young maize seedlings with a single primary root, shortly after imposing a defined water deficit regime [addition to hydroponic root medium of a nonpenetrating osmolyte, poly (ethylene glycol) 6000 (PEG) at –0.5-MPa water potential)]. Water deficit rapidly inhibited leaf growth and this inhibition was maintained for hours and days. The water deficit treatment also induced (within minutes) associated decreases in the extensibility characteristics of the expanding cell walls in the leaf elongation zone (Chazen and Neumann 1994). Most importantly in the present context, similar leaf responses were observed when the seedling roots were killed by freeze–thaw treatment with liquid nitrogen prior to imposing water deficit, i.e., prior to PEG addition. PEG does not penetrate the cell walls of live or killed roots and effectively decreases water availability in each case (Chazen et al. 1995). Supportive findings were obtained in additional experiments on wheat seedlings (Neumann et al. 1997). An inescapable

conclusion is that any neurobiological activity of a diffuse brain in the root apex would have been disrupted by freeze–thaw treatment of the root and was apparently unnecessary for the induction of the leaf growth inhibition by water deficit. Similarly, the freeze -thaw treatment would have disrupted the phloem transport system in the root so that active signal transmission via phloem-related tissues acting as “nerves” was prevented. Chazen and Neumann (1994) concluded that neither electrical nor chemical signals from the roots were essential for the rapid root-to-leaf transmission of information concerning root water status. Instead they proposed that passive hydraulic signaling via the xylem transport system was involved. Rapid hydraulic signaling via pressure changes in the xylem may in this case represent an analogue to animal nervous systems. However, root apex “brains” are not required for such signaling.

In another set of experiments Snir and Neumann (1997, and unpublished data) investigated the short-term effects of altered mineral nutrient supply on leaf growth in maize seedlings with a single primary root about 8 cm in length, a caryopsis and an emerging first true leaf. The interactive effect of excising a 1.5 cm section from the root apex was investigated with the aim of determining whether upward transport of electrical or chemical signals (e.g., plant hormones such as cytokinins) from the root tip might be involved in regulating leaf growth. Leaf growth was measured for 15 h following partial root excision and under different nutrient supply regimes. Root excision caused a reduction in subsequent leaf growth in seedlings continuously supplied with mineral nutrient solution (Table 5.2). These findings suggest that for seedlings supplied with mineral nutrient solution, root tip excision may have prevented the generation and transport to the shoot of growth essential hormone(s) or electrical signals from the root tip. Alternatively, tip excision simply reduced root length and surface area, thereby limiting rates of uptake and transport to the leaf growing zone of growth-essential mineral nutrients.

When seedlings were grown on a minimal physiological solution (1 mM $CaCl_2$ without additional mineral nutrients) leaf growth was slightly reduced but root tip excision had no additional growth inhibitory effect. The nutrients required for the ongoing growth of leaves in the low-nutrient treatment were presumably transported from reserves in the caryopsis. The fact that excision of the root apex from low-nutrient seedlings had no inhibitory effect on leaf growth indicates that hormonal or electrical signals from any diffuse brain in the root tip were not essential for the maintenance of leaf growth under these conditions.

In a third experiment, relationships between water deficit and growth were studied in succulent, photosynthetic stem cuttings of *Hylocereus undatus* ((Haworth) Britton and Rose), a vine cactus from central America (Nerd and Neumann 2004). This species is grown as a fruit crop in Israel

Table 5.2. Effects of root tip removal in the presence or absence of mineral nutrient supply on growth of maize seedling primary leaves. Maize seeds (*Zea mays* L., cv 646) were germinated for 2 days in the dark and then transferred to hydroponic culture for 2 days under the conditions of mineral nutrient supply indicated. The length of the emerging first true leaves was assayed for 15 h with and without excision of a 1.5-cm section from the root apex. Initial root length about 8 cm. Means ± standard error, $n = 10$ (Snir and Neumann, previously unpublished data)

	Leaf elongation (cm 15 h^{-1})	
	Intact root	Root minus tip
With mineral nutrients	2.19 ± 0.06	1.93 ± 0.07
Without mineral nutrients	1.60 ± 0.03	1.63 ± 0.06

and the experiments were originally designed to investigate the relationships between root irrigation frequency and shoot growth. When cuttings of mature stems (about 30 cm in length) were held in well-watered sandy soil they produced basal root systems and new apical stems. The rooted cuttings were then subjected to drought conditions, i.e., cessation of irrigation. During the first 5 days of drought treatment, the soil and roots rapidly dried as soil water potentials decreased from −0.02 to −1.50 MPa. However, despite the rapid drying of soil and roots, the growth of young apical stems (2–3 cm long) emerging from the mature stems only decreased significantly after 3 weeks of drought. Even then, the water content of the growing stems was decreased by only 2% and their bulk water potentials were not significantly different from those of the irrigated control stems (at about −0.40 MPa). At the same time, water content in drought-treated mature stems decreased by 8% and their water potentials decreased from −0.55– −0.80 MPa. Thus, water reserves appeared to be moving from the fully expanded mature stems to the young growing stems. Several lines of evidence indicated that active phloem transport of water (and associated solutes) from mature to developing stems was the mechanism which allowed the maintenance of new growth despite the complete drying of soil and roots: (1) phloem girdling at the base of the growing stems inhibited their elongation; (2) secretion of sucrose-containing nectar by growing stems was maintained during drought; (3) the water potential gradient was in the wrong direction for any xylem transport of water from mature to growing stems and axial hydraulic conductivities for xylem transport into young stems were either zero or comparatively low.

An important point to note is that the water potentials and growth of the young stems were maintained at prestress levels for weeks after the soil and roots had become dry. Thus, root apex “brains” and root vascular tissues were clearly not required during the sustained diversion of

resources from mature to young stems that facilitated the maintenance of growth. Conversely, the fact that a phloem girdle between mature and young stems inhibited the growth of the young stems reinforces the importance of phloem transport tissues in interorgan communication. Although root functioning did not appear to be essentially involved in stem growth regulation, phloem transport clearly was. The growth regulatory function of phloem in this case could again be considered analogous to the signaling facilitated by the nerves of animals. However, the 3-week time scale required for this shoot growth response to relatively rapid dehydration of the root environment to become established was totally different from the seconds required for most animal responses to environmental changes.

5.5 Conclusions and Future Perspectives

Some support is provided in this chapter for the drawing of analogies between the signaling roles of plant long-distance transport systems and animal nervous systems. However, the specific findings reviewed here do not indicate that root apices function as essential neurobiological command centers involved in regulating shoot growth responses to adverse changes in the root environment. Cell-to-cell signaling via plasmodesmatal connections and variations in long-distance transport of hormones, essential nutrients and water via vascular tissues may conceivably provide the regulation needed to integrate most higher-plant growth responses to environmental changes.

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6 Signals and Targets Triggered by Self-Incompatibility in Plants: Recognition of "Self" Can Be Deadly

S.G. Thomas, S. Huang, C.J. Staiger, V.E. Franklin-Tong

Abstract Plants often respond to stimuli with dramatic rearrangements of their actin cytoskeleton. Identification of the signals and transduction machinery that lead to changes in actin dynamics and organization is therefore of considerable interest. The self-incompatibility (SI) response in the field poppy, *Papaver rhoeas* L., involves signal-mediated inhibition of pollen tube growth in response to interaction with incompatible S proteins. This triggers larges increases in $[Ca^{2+}]_i$ and downstream a number of signalling components and targets are modified in incompatible pollen. We have observed dramatic alterations to the actin cytoskeleton in response to SI and established that this involves massive and sustained actin depolymerization. We have begun to identify and characterize several actin-binding proteins, including profilin and gelsolin, that may cooperate to transduce the signal from a Ca^{2+} wave into destruction of the cytoskeletal network that is essential for tip growth. Recently, we identified a role for programmed cell death (PCD) signalling cascades being triggered in SI and that a caspase-like activity is involved in mediating irreversible pollen tube inhibition. Our data suggest that there is evidence for crosstalk between the SI-induced signalling cascades. We are currently investigating whether the signalling cascades for actin alterations and PCD are linked and whether the actin cytoskeleton functions as a sensor of cellular stress and can initiate PCD. Our current knowledge of the signalling cascade in *P. rhoeas* pollen therefore involves both early and late responses that work in concert to ensure that pollen does not effect fertilization. Early cessation of tip growth is mediated by destruction of the actin cytoskeleton, and this appears to cross-talk with a subsequent PCD cascade that commits the pollen to die.

6.1 Introduction

Cell signalling mechanisms are vital for plants to adapt and survive in a harsh and challenging environment (Delledonne et al. 2005; Nürnberger et al. 2005; Rhodes et al. 2005 in this volume). Plants respond to various stimuli with dramatic rearrangements of their cytoplasm and these are often mediated by a dynamic actin cytoskeleton. Thus, a major focus of research is the identification of the signals and transduction machinery that lead to changes in actin dynamics and organization.

The self-incompatibility (SI) response in the field poppy, *Papaver rhoeas* L., provides a good example of a genetically controlled system that employs signal-mediated inhibition of pollen tube growth in response to a specific stimulus. The ability to reproduce the SI response in *Papaver* pollen in

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vitro (Franklin-Tong et al. 1988) has allowed dissection of the signalling cascades triggered by SI (Franklin-Tong and Franklin 2003). Here we describe dramatic alterations to the actin cytoskeleton that occur during SI and the characterization of several actin-binding proteins (ABPs) that may play central roles. We also show that SI involves signalling to programmed cell death (PCD) cascades. We have begun to explore whether the signalling cascades for actin alterations and PCD are linked and whether the actin cytoskeleton functions as a sensor of cellular stress and can initiate PCD.

6.1.1 Pollen–Pistil Interactions

Sexual reproduction is an excellent example of a process whereby plant cells respond to many different stimuli and utilize extensive signalling cascades. Pollination involves the deposition of pollen grains on a receptive stigma. This triggers numerous pollen–pistil interactions, including hydration of the pollen grain, germination and directional growth of the pollen tube through the pistil, and delivery of the sperm cells to the ovule, where they effect fertilization resulting in seed production. These events are tightly controlled at both the genetic and the biochemical level. Two recent reviews discuss pollination in more detail (Edlund et al. 2004; Sanchez et al. 2004).

The pollen tube elongates via tip growth, which involves the precise control of exocytosis and delivery of new plasma membrane and cell wall materials to the tube apex. It responds to chemical and physical signals during its journey to the ovule (reviewed by Franklin-Tong 1999a). For example, signals from the style include arabinogalactan proteins (Cheung et al. 1995; de Graaf et al. 2003) triacylglycerides (Wolters-Arts et al. 1998), chemocyanin (Kim et al. 2003), and γ-amino butyric acid (GABA) (Palanivelu et al. 2003). These signal molecules are believed to be involved in the guidance of the pollen tube through the pistil. Within the pollen tube, cytosolic free calcium ($[Ca^{2+}]_i$) has been shown to be an important second messenger regulating pollen tube growth (reviewed by Franklin-Tong 1999b). Growing pollen tubes exhibit a tip-focused $[Ca^{2+}]_i$ gradient (Rathore et al. 1991; Miller et al. 1992) that oscillates and is coordinated with growth (Holdaway-Clarke et al. 1997; Messerli et al. 1999, 2000). Although our understanding of this signalling cascade is incomplete, it is clear that $[Ca^{2+}]_i$ oscillations allow spatio-temporal control of key processes involved in pollen tube growth (reviewed by Holdaway-Clarke and Hepler 2003).

6.1.2 Self-Incompatibility

One aspect of pollen–pistil interactions that has been the focus of much research is SI. This is a genetically controlled mechanism which allows plants to prevent inbreeding. Essentially, SI is a cell–cell recognition system that allows the discrimination of genetically related pollen ("self" or incompatible pollen) from genetically unrelated pollen ("non-self" or compatible pollen). For detailed reviews on the different types of SI, the reader is directed to three recent reviews (Franklin-Tong and Franklin 2003; Hiscock and McInnis 2003; Kao and Tsukamoto 2004).

In *Papaver* pollen, the cellular and molecular bases of the SI system have been investigated in detail (Franklin-Tong and Franklin 2003). The pistil component, the S protein (Foote et al. 1994), is a small (about 15-kDa) secreted protein which acts as a ligand for the pollen component, which is thought to be a plasma membrane receptor (Franklin-Tong and Franklin 2003). During an incompatible interaction, several events are triggered in the pollen. A rapid influx of extracellular Ca^{2+} and large increases in $[Ca^{2+}]_i$ precede the dissipation of the tip-focused $[Ca^{2+}]_i$ gradient, and comprise the initial second messenger signals to downstream targets (Franklin-Tong et al. 1993, 1995, 1997, 2002). These targets include the rapid reorganization and depolymerization of F-actin (Geitmann et al. 2000; Snowman et al. 2002), which we discuss in more detail later. SI also involves the activation of several protein kinases leading to the phosphorylation of a number of proteins. These include p26, a soluble inorganic pyrophosphatase (Rudd et al. 1996; Rudd and Franklin-Tong 2003). Phosphorylation of p26 and the inhibition of its activity by increases in $[Ca^{2+}]_i$ are believed to interfere with pollen tube growth. A mitogen-activated protein (MAP) kinase, p56, is also phosphorylated (Rudd and Franklin-Tong 2003; Rudd et al. 2003). The timing of p56 activation (Rudd et al. 2003), after arrest of pollen tube growth, suggests that p56 is not involved in tip-growth inhibition, but instead may signal to events occurring downstream. SI also triggers PCD (Jordan et al. 2000; Thomas and Franklin-Tong 2004) and this is discussed later.

6.2 The Actin Cytoskeleton and Self-Incompatibility

6.2.1 Actin as a Sensor of Environmental Stimuli

A network of polymers, the actin cytoskeleton, provides a dynamic framework for multiple cell functions. At the protein level, it is composed of actin filaments (F-actin) polymerized from monomeric, globular actin (G-actin). The actin cytoskeleton has been shown to play an essential role in numerous processes, including cell motility, organelle movement, vesicle trafficking and cytoplasmic streaming (reviewed by McCurdy et al. 2001; Staiger and Hussey 2004). Actin filaments reorganize in response to various signalling events and this mediates diverse responses to external environmental cues. A major focus of research on the cytoskeleton is the dissection of signalling cascades that regulate actin dynamics (reviewed by Staiger 2000). In incompatible *Papaver* pollen, F-actin reorganizes rapidly (Geitmann et al. 2000; Snowman et al. 2002). Actin rearrangements in response to specific stimuli have also been described in stomatal guard cells responding to abscisic acid and light (Eun and Lee 1997), root hairs responding to Nod factors from *Rhizobium* bacteria (Cardenas et al. 1998; Miller et al. 1999) and epidermal cells responding to fungal and oomycete pathogens (Kobayashi et al. 1992, 1993).

The dynamic nature of the actin cytoskeleton depends on the spatial distribution and the local activity of a complex mixture of ABPs. The number of ABPs identified in plants is growing rapidly and includes profilin, actin-depolymerizing factor (ADF)/cofilin, villin/gelsolin-like proteins, fimbrin, the Arp2/3 complex, AIP1, EF1α, and capping protein (reviewed by Staiger and Hussey 2004). The activity and regulation of these ABPs by second messengers suggest that they play key roles as transducers of extracellular stimuli; however, an involvement in specific cellular signalling processes has yet to be demonstrated.

Pollen tube tip growth is particularly amenable to studying the signals responsible for mediating changes to the actin cytoskeleton. It is widely assumed that the actin cytoskeleton helps guide or regulate the delivery of secretory vesicles to the pollen tube apex, but exactly how this is accomplished remains to be established. Furthermore, it is thought that the control of pollen tube growth involves a complex interplay between the cytoskeleton and signalling cascades, although there is currently little direct evidence for this. However, early studies showed that increasing $[Ca^{2+}]_i$ artificially can disrupt actin filaments in pollen tubes (Kohno and Shimmen 1987, 1988), suggesting that Ca^{2+} signals to alterations in actin organization. Analysis of a family of Rho-related GTPases in plants (ROPs) (reviewed

by Yang 2002) has provided significant new insights into the interactions between Ca^{2+} signals and actin dynamics. It has been demonstrated that the pollen-expressed ROP1, through its interacting partners RIC3 and RIC4, plays a role in two signalling cascades: one involving Ca^{2+} signalling, the other involving actin dynamics (Gu et al. 2003, 2005). These data begin to explain how Ca^{2+} signals are linked to the spatio-temporal control of actin dynamics and tip growth in pollen tubes.

It is well established that both actin organization and levels of F-actin modulate pollen tube tip growth (Gibbon et al. 1999; Geitmann and Emons 2000; Fu et al. 2001; Vidali et al. 2001; Lovy-Wheeler et al. 2005). A variety of cytological approaches have allowed researchers to build a consensus view of the distribution of the actin cytoskeleton in growing pollen tubes. There are three "zones" of F-actin in the pollen tube: long arrays of longitudinal actin filament bundles in the shank; a dense sub-apical meshwork of F-actin in a "basket-like" or ring configuration; and at the tip, a fine array of dynamic filaments. It is likely that the axial cables support reverse-fountain-pattern cytoplasmic streaming and that the apical arrays regulate secretory vesicle trafficking. Figure 6.1a shows this arrangement for *Papaver* pollen tubes.

6.2.2 Actin as a Target for Self-Incompatibility Signals in Incompatible Pollen

Evidence that SI signals to the actin cytoskeleton was first provided by the observation that dramatic alterations to F-actin are triggered by SI induction in incompatible pollen tubes (Geitmann et al. 2000; Snowman et al. 2002). Detectable alterations were extremely rapid, occurring within 1 min. The distinctive sub-apical basket-like configuration disappeared and a large "blob" of F-actin appeared at the pollen tube apical region (Fig. 6.1b). The overall intensity of phalloidin staining was reduced substantially, indicating a loss of F-actin, and by 5–10 min after SI induction, the longitudinal F-actin bundles had largely disappeared (Fig. 6.1c,d). The remaining F-actin had a fine, speckled appearance (Fig. 6.1d,e), suggesting severing or depolymerization of F-actin and pronounced cortical F-actin was evident (Fig. 6.1c–e). Further alterations were evident between 10 and 20 min; F-actin appeared as large aggregates or "punctate foci" (Fig. 6.1e,f) and these persisted for at least 3 h. These alterations were specific to induction of SI in incompatible pollen tubes, as control pollen tubes showed no changes to actin organization. Furthermore, the SI-induced rearrangement of actin was demonstrated to be independent of growth arrest (Geitmann et al. 2000).

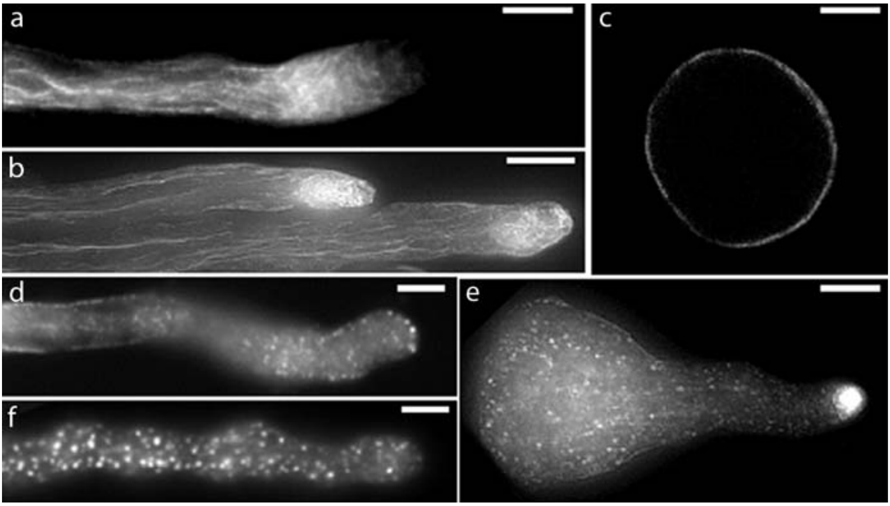

Fig. 6.1. Self-incompatibility (*SI*) induces dramatic rearrangements of actin cytoskeleton organization in *Papaver* pollen. **a** The actin cytoskeleton of a normally growing pollen tube has longitudinal arrays of F-actin in the pollen tube shank, a dense sub-apical network of F-actin and a fine array of dynamic filaments at the tip. **b** Following SI induction rapid reorganization is observed. Within 1 min, F-actin accumulates in the tip of the pollen tube. **c,d** By 20 min after SI induction, the F-actin has disappeared from the cortex and is localized to the periphery of grains and pollen tubes and "punctate foci" of F-actin begin to appear. **e,f** The punctate foci persist for greater than 60 min and increase in size over time. **a,b,d–f** are epifluorescence microscope images of rhodamine–phalloidin-labelled pollen tubes. **c** is a single optical section from confocal microscope imaging of rhodamine–phalloidin-labelled pollen grain. *Bars* represent 10 μm

6.2.3 Self-Incompatibility Stimulates Rapid and Sustained Depolymerization of F-Actin

Because the actin reorganization observed during the early stages of the SI response suggested that actin depolymerization had occurred, a quantitative approach was used to investigate this further. A technique based on the number of fluorescent-phalloidin binding sites (Howard and Oresajo 1985; Lillie and Brown 1994) was used to determine the concentration of actin in filamentous form in pollen (Gibbon et al. 1999; Snowman et al. 2002). We found that the levels of F-actin in pollen throughout hydration, germination and early growth were constant (Snowman et al. 2002). Upon SI induction, a rapid and sustained decrease in F-actin levels was induced. Within 1 min there was a significant reduction in F-actin. These levels continued to decrease, so that by 5 min after SI induction, F-actin levels were less than 50% of the controls and by 1 h were reduced by 74% (Snowman et

al. 2002). Thus, SI induces massive and sustained F-actin depolymerization in incompatible pollen.

6.2.4
Increases in Cytosolic Calcium Lead to Changes in F-Actin

As we had established that Ca^{2+} acts as a second messenger during the SI response (Franklin-Tong et al. 1993, 1997), we wondered whether F-actin was a target for Ca^{2+}-dependent signalling. We therefore investigated whether increasing $[Ca^{2+}]_i$ in pollen tubes, using the Ca^{2+} ionophore A23187 and mastoparan (Franklin-Tong et al. 1993, 1996), stimulated changes in F-actin. These treatments triggered reorganization of the F-actin cytoskeleton that appeared broadly similar to those stimulated by SI and led to quantitative reductions in F-actin levels (Snowman et al. 2002). This suggests that during SI increases in $[Ca^{2+}]_i$ perturb the actin cytoskeleton.

6.2.5
Profilin and Gelsolin: Mediators of Actin Alterations?

The marked changes to the actin cytoskeleton in the SI response raised the question of what molecular machinery transduces the changes in $[Ca^{2+}]_i$ into destruction of the F-actin. ABPs are stimulus-response modulators of actin cytoskeleton reorganization (reviewed by Holdaway-Clarke and Hepler 2003; Staiger and Hussey 2004). F-actin depolymerization could result from a loss of side-binding proteins, capping of filament ends, stimulation of filament severing, increased activity of a sequestering protein, or a combination of these activities. However, few ABPs in pollen tubes have been well characterized (reviewed by Hepler et al. 2001; McCurdy et al. 2001; Staiger and Hussey 2004). Because actin depolymerization can be achieved using calcium ionophores, we attempted to model this in vitro with Ca^{2+}-sensitive ABPs. Although plant ADF/cofilin can cause actin depolymerization and is regulated by Ca^{2+} indirectly, increases in $[Ca^{2+}]_i$ are expected to inhibit the F-actin depolymerizing activity of ADF (Smertenko et al. 1998, Allwood et al. 2001), so its involvement in SI has not been examined.

Pollen profilin exhibits increased actin-sequestering activity at elevated, but physiologically relevant, Ca^{2+} concentrations (Kovar et al. 2000). A large pool of profilin was predicted to buffer G-actin and maintain the low actin polymer level in pollen (reviewed by Staiger and Hussey 2004). However, experiments with native *Papaver* pollen profilin failed to account for the high level of depolymerization observed during SI (Snowman et al. 2002). We proposed that other ABPs may function in concert with profilin to achieve sustained actin depolymerization during SI.

6.2.6
PrABP80 is Poppy Gelsolin

PrABP80 is a Ca^{2+}-regulated ABP which may be involved in SI-mediated actin depolymerization (Huang et al. 2004). PrABP80 was originally identified by DNase I–Sepharose chromatography during attempts to isolate native poppy pollen actin. Tandem mass spectrometry sequence analysis and kinetic actin polymerization assays showed that it belongs to the villin/gelsolin family. Furthermore, the native protein was demonstrated to have potent Ca^{2+}-dependent severing activity in vitro (Huang et al. 2004). As shown in Fig. 6.2, PrABP80 significantly reduced the length of pre-formed actin filaments after incubation in the presence of 160 μM free Ca^{2+} for 30 min (Fig. 6.2b) compared with incubation in the presence of 15 nM free Ca^{2+} (Fig. 6.2a). It was also shown directly with time-lapse fluorescence imaging that PrABP80 could sever actin filaments in a Ca^{2+}-dependent manner. PrABP80 could, therefore, assist F-actin depolymerization by Ca^{2+}-activated severing and the creation of new pointed ends for depolymerization by profilin. In addition, PrABP80 caps the barbed end of actin filaments, thereby blocking assembly of the profilin–actin complex and allowing profilin to function as a simple actin sequestering protein. The feasibility of such a mechanism was supported by experiments in which equimolar amounts of profilin were added to pre-existing actin filaments together with nanomolar amounts of PrABP80. In these assays, polymer levels were monitored by fluorescence of pyrene-labelled actin in the fluorimeter. The data from a typical experiment in the presence of 1 μM free Ca^{2+}, which mimics the $[Ca^{2+}]_i$ observed in pollen tubes during the SI response, are shown in Fig. 6.2c. The extent and rate of actin depolymerization were dramatically increased by substoichiometric amounts of PrABP80, when compared with profilin alone; moreover, significant depolymerization was not observed when free Ca^{2+} was reduced to 15 nM (Fig. 6.2c).

Thus, we have identified a potential mechanism that links SI-induced $[Ca^{2+}]_i$ changes to actin depolymerization. We propose that PrABP80 functions at the centre of the SI response by creating new filament pointed ends for disassembly and by blocking barbed ends from profilin–actin assembly. To test this molecular model thoroughly, we will need to determine the cellular concentrations for PrABP80 in pollen, measure its affinity for plant actin filament ends, and determine the efficiency of severing at physiological $[Ca^{2+}]_i$.

The actin depolymerization observed during SI is somewhat unusual, since the level and extent of depolymerization are far greater than those required to inhibit growth. Further alterations to the actin cytoskeleton continue long after the inhibition of tip growth, suggesting that SI-induced

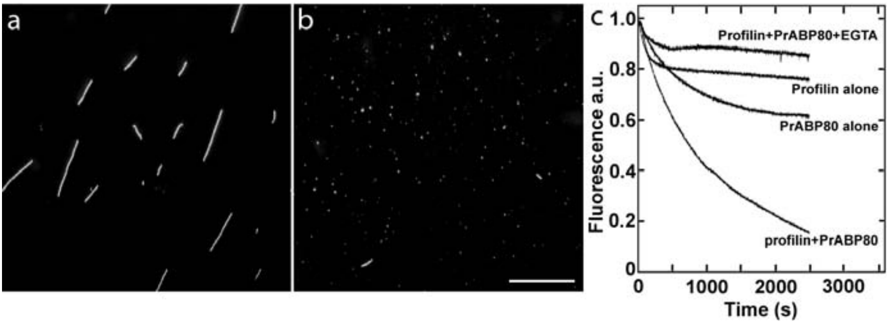

Fig. 6.2. PrABP80 has potent calcium-dependent F-actin severing activity. **a,b** Actin filaments (4 μM) labelled with rhodamine–phalloidin were incubated with PrABP80 in the presence of 15 nM free Ca^{2+} (**a**) or 160 μM Ca^{2+} (**b**) and images were collected after 30-min incubation. PrABP80 significantly reduced the length of actin filaments (mean 0. 4±0.4 μm) after incubation in the presence of 160 μM free Ca^{2+} (**b**). Reactions performed in the presence of 15 nM free Ca^{2+} had a mean filament length of 4. 7±3.2 μm (**a**). The *bar* represents 10 μm. **c** The severing activity of PrABP80 was also shown by enhancement of profilin-mediated depolymerization in the presence of 1 μM free Ca^{2+} as monitored by fluorescence of pyrene actin. *Zea mays* profilin alone (ZmPRO5; 2.5 μM) or ZmPRO5 together with PrABP80 were added to an F-actin solution prepared from 2.5 μM actin (40–50% pyrene-labelled). Depolymerization was recorded as a reduction in pyrene fluorescence (arbitrary units) beginning from the addition of protein at time zero. The extent and rate of depolymerization were increased by substoichiometric amounts of PrABP80 + profilin (*bottom curve*), compared with profilin alone or PrABP80 alone (*middle curves*). Moreover, a similar level of depolymerization was not observed when free calcium was reduced to 15 nM, a condition where PrABP80 should function like a barbed-end cap (*top curve*)

signalling events continue after inhibition of growth. We therefore believe that some of these events may act to make growth arrest irreversible. In yeast and some animal cells, sustained actin depolymerization is observed during apoptosis and the actin cytoskeleton has been shown to play a functional role in the initiation of PCD by modulating PCD signalling cascades. (Kayalar et al. 1996; Levee et al. 1996; Janmey 1998; Korichneva and Hammerling 1999; Rao et al. 1999; Morley et al. 2003; Gourlay et al. 2004; Gourlay and Ayscough 2005). We noted that the sustained actin depolymerization observed in incompatible pollen was highly reminiscent of apoptosis. This suggested that a PCD signalling cascade might be triggered by SI in incompatible pollen.

6.3 Programmed Cell Death and Self-Incompatibility

6.3.1 Key Features of Programmed Cell Death

PCD is a conserved process used to remove unwanted cells in plants and animals during development and in response to external stimuli. In animal cells, apoptosis (a form of PCD) can be divided into initiation and execution phases. During the initiation phase, signalling cascades are triggered that prepare the cell for death. Mitochondria are a key target for the initial signalling cascade. After receiving appropriate signals, mitochondria become depolarized and cytochrome c is released from the mitochondrial intermembrane space into the cytosol (Jiang and Wang 2004; Yao et al. 2004). In animal cells, cytosolic cytochrome c forms the apoptosome, a protein complex that activates executioner caspases, the key proteases involved in cell death (reviewed by Strasser et al. 2000). Cytochrome c release is therefore a classic marker of PCD in many organisms (Adrain and Martin 2001).

Caspases are proteases that cleave target proteins after an aspartate residue (reviewed by Riedl and Shi 2004). They are present as inactive zymogens and are activated rapidly during apoptosis. Caspase-3 is the main executioner protease activated during apoptosis and is responsible for cleavage of many cellular proteins (reviewed by Fischer et al. 2003). Various tetra-peptide inhibitors of caspases are available and these have aided the study of caspase-dependent apoptosis. Caspase-3 has the recognition sequence DxxD and the caspase-3 inhibitor, DEVD, is based on this. Inhibition of protease activity using DEVD implicates the involvement of a caspase-3 like activity. The peptide YVAD is often used to demonstrate specificity. The nuclear DNA repair protein, poly(ADP-ribose) polymerase (PARP), was one of the first caspase-3 substrates to be identified (Lazebnik et al. 1994) and is cleaved into 89- and 24-kDa fragments during apoptosis. Caspases also cleave endogenous nuclease inhibitors, which activates nucleases and leads to the fragmentation of nuclear DNA (Nagata 2000). Thus, the identification of cleavage fragments of a known caspase substrate, the inhibition of a caspase-like activity using DEVD and an increase in the incidence of DNA fragmentation are all considered diagnostic for apoptosis.

PCD in plants is less well studied compared with PCD in animals, but many examples of developmental PCD (reviewed by Kuriyama and Fukuda 2002) and PCD in response to external stimuli have been identified. These include pathogen attack (Lam et al. 2001; Greenberg and Yao 2004), temperature stress (Swidzinski et al. 2002), reactive oxygen species (Clarke et al. 2000; Overmyer et al. 2005) and UV radiation (Danon et al. 2004). However,

many of the genes encoding components of the apoptotic machinery, including caspases, have either not yet been identified in plants or are simply not present (reviewed by Woltering et al. 2002). Nevertheless, there is no doubt that PCD occurs in plants and much of the evidence for it has come from biochemical studies (reviewed by van Doorn and Woltering 2005). We have used markers of PCD to investigate whether an incompatible SI response triggers PCD in *Papaver* pollen.

6.3.2 Programmed Cell Death is Triggered During the *Papaver* Self-Incompatibility Response

Cytochrome c release is an early event of SI-induced PCD. Since leakage of cytochrome c into the cytosol is a key early marker for PCD, we investigated whether this occurred in pollen tubes undergoing incompatible SI. We showed that large increases in cytosolic cytochrome c were detected in incompatible pollen (Thomas and Franklin-Tong 2004). The increase in cytochrome c release was rapid, beginning at 10 min and increasing up to 2 h, after SI induction. In controls, no release of cytochrome c was observed. The first detection of cytochrome c in the cytosol at around 10 min corresponds to the point at which peak phosphorylation and MAP kinase activity of p56 are detected (Rudd et al. 2003). Our data may therefore indicate crosstalk between different signalling cascades and may represent the point at which the pollen tube becomes committed to death.

DNA fragmentation is stimulated by SI. Since DNA fragmentation is a classic marker for PCD, we investigated whether DNA fragmentation occurred in incompatible *Papaver* pollen undergoing SI. DNA fragmentation was detected in incompatible pollen and was S-specific, as it was not observed in either untreated pollen tubes or compatible pollen tubes (Jordan et al. 2000). DNA fragmentation is first detected at 4 h and continues to increase for at least 16 h after SI induction. Pre-treatment of pollen tubes with DEVD prior to the induction of SI caused a significant reduction in the amount of DNA fragmentation, reducing it from levels of 71.6% in SI to 18.97% in pollen tubes with SI-induced in the presence of DEVD (Fig. 6.3a) (Thomas and Franklin-Tong 2004). YVAD (used as a negative control) displayed only a small decrease in DNA fragmentation. These data implicate the involvement of a caspase-3 like activity in SI-mediated DNA fragmentation. Furthermore, DEVD pre-treatment of pollen tubes allowed growth to resume following SI induction, whilst YVAD did not alleviate SI-induced growth inhibition (Thomas and Franklin-Tong 2004). These data demonstrate that a caspase-3 like activity is also involved in mechanisms mediating SI-induced pollen tube inhibition.

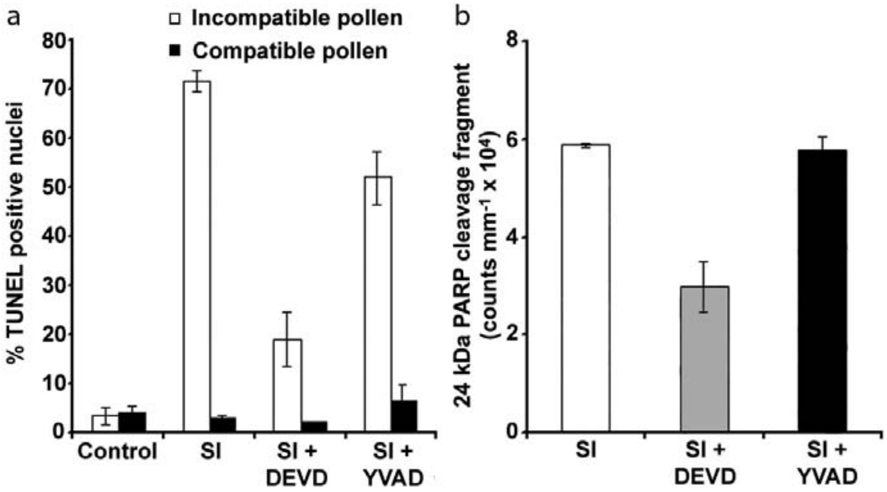

Fig. 6.3. A caspase-like activity is triggered during SI-induced programmed cell death (*PCD*). **a** DNA fragmentation was assayed in pollen tubes following SI induction, using terminal deoxynucleotide transferase dUTP nick end labelling (*TUNEL*). In untreated pollen (control) and pollen challenged with compatible S proteins (*black bars*), low levels of DNA fragmentation were observed. In incompatible pollen (SI, *white bar*) high levels of DNA fragmentation levels were observed. This was reduced by pre-treatment of pollen with the peptide DEVD (SI + DEVD, *white bar*), whereas YVAD (SI + YVAD, *white bar*) gave only a small decrease in DNA fragmentation. *Bars* are the mean ± the standard error of the mean (*SEM*). **b** Poly(ADP-ribose) polymerase (*PARP*) cleavage activity in pollen protein extracts was assayed by adding bovine PARP and performing Western blotting. Incompatible pollen protein extracts 5 h after SI induction (SI, *white bar*) showed high PARP cleavage activity. This activity was reduced in the presence of DEVD (SI + DEVD, *grey bar*), whereas YVAD (SI + YVAD, *black bar*) had no effect. *Bars* are the mean ± the SEM

SI stimulates a caspase-3 like cleavage activity. We examined more directly whether SI stimulated a caspase-3 like activity by testing whether incompatible pollen had an activity that could cleave PARP. Using bovine PARP as a substrate, we demonstrated that protein extracts from incompatible pollen tubes had an activity that generated a 24-kDa PARP cleavage product (Thomas and Franklin-Tong 2004). Extracts from untreated and compatible pollen did not have a PARP-cleavage activity, demonstrating S-specificity. Pre-treatment of extracts with DEVD gave 60% less 24-kDa PARP cleavage product, whilst no effect of YVAD pre-treatment was observed (Fig. 6.3b). This clearly implicates the involvement of a caspase-3 like activity, induced in incompatible pollen by SI, in the generation of the 24-kDa PARP cleavage fragment.

Increases in Ca^{2+} **stimulate PCD in *Papaver* pollen.** Since $[Ca^{2+}]_i$ acts as a second messenger for the SI response, we investigated if increases in

$[Ca^{2+}]_i$ may signal to PCD in *Papaver* pollen. Treatment of pollen tubes with mastoparan (Franklin-Tong et al. 1996) stimulated large increases in cytochrome c release into the cytosol within 5 min (Thomas and Franklin-Tong 2004). These data showed that increases in $[Ca^{2+}]_i$ in pollen act upstream of cytochrome c release. Mastoparan-stimulated pollen extracts also had high PARP-cleavage activity and the calcium channel blocker, La^{3+} (Franklin-Tong et al. 2002), gave a 70% decrease in this activity (Thomas and Franklin-Tong 2004), indicating that increases in $[Ca^{2+}]_i$ can trigger a caspase-3 like activity. Together these data suggest that the Ca^{2+} signals stimulated by SI are part of the signalling cascade responsible for triggering PCD in incompatible pollen.

6.3.3
A Link Between Actin and Programmed Cell Death?

The F-actin depolymerization observed during SI certainly inhibits pollen tube growth. However, the extent of depolymerization is much greater than the level required to stop tip growth. This suggested additional functions for SI-induced actin depolymerization. As discussed earlier, in animal and yeast cells there is evidence for actin dynamics signalling to PCD, but no-one has investigated this possibility in plants yet. We have explored a possible link between actin depolymerization and PCD, using the G-actin binding reagent latrunculin B to depolymerize pollen tube F-actin and then measured PCD using DNA fragmentation. Preliminary data indicate that modest and transient reductions in actin polymer levels are sufficient to trigger caspase-3 mediated PCD in *Papaver* pollen tubes (Thomas, Huang, Staiger and Franklin-Tong, unpublished data). Thus, there is evidence for changes in actin dynamics initiating PCD in *Papaver* pollen. This suggests crosstalk exists between the actin and PCD signalling networks in pollen.

6.4
Conclusions and Future Perspectives

Some of the major components and events that play a role in mediating SI in *Papaver* pollen have now been identified. Figure 6.4 shows a model that highlights current knowledge about the signals and targets triggered by SI in incompatible pollen. These include Ca^{2+}, actin, PCD, and other components not discussed in detail here. Following SI induction, which we assume involves the interaction of the stigmatic S protein with its cognate pollen S receptor, there is a rapid influx of Ca^{2+} accompanied by increases in $[Ca^{2+}]_i$ in the pollen tube. The tip-focused $[Ca^{2+}]_i$ gradient dissipates and

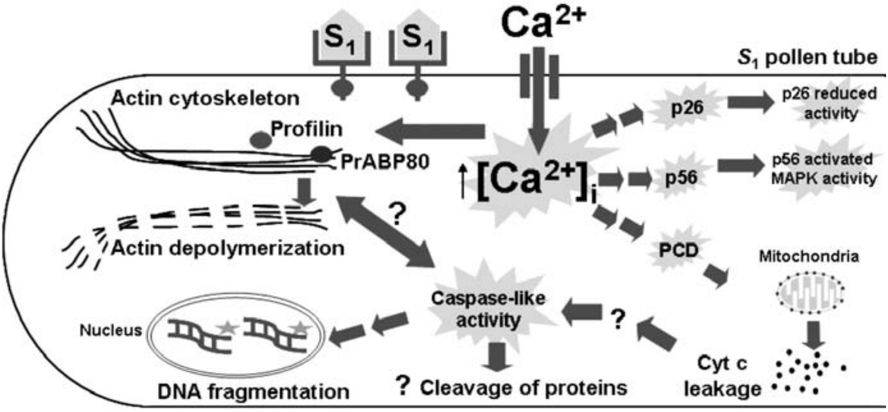

Fig. 6.4. Signals and targets involved in SI in incompatible *Papaver* pollen. S proteins (S_1) bind to a specific pollen S receptor on an S_1 pollen tube during an incompatible SI interaction. This stimulates influx of Ca^{2+} and increases in $[Ca^{2+}]_i$, which triggers an intracellular Ca^{2+} signalling cascade in the pollen tube. Depolymerization of F-actin is likely to be mediated by Ca^{2+}-regulated actin-binding proteins, including profilin and PrABP80. SI also tri ggers PCD in incompatible pollen. Hallmarks of PCD, including cytochrome c leakage from mitochondria, a caspase-3 like cleavage activity and caspase-mediated DNA fragmentation have been observed in incompatible pollen. There is preliminary evidence for altered actin dynamics or polymer levels acting as the trigger for PCD, which commits the pollen to die. Other SI-triggered signalling events in incompatible pollen include rapid increases in the Ca^{2+}-dependent phosphorylation of a number of proteins, including p26, a soluble inorganic pyrophosphatase and p56, a mitogen-activated protein kinase (*MAPK*)

tip growth is inhibited within a few minutes. We have shown that $[Ca^{2+}]_i$ acts as a second messenger and triggers a number of downstream events (Fig. 6.4). Because the tight spatio-temporal regulation of actin dynamics in pollen tubes is crucial for growth, the cytoskeleton is an ideal target for the Ca^{2+}-signalling cascade during SI. Changes to the cytoskeleton during SI are probably mediated through the cooperative action of several Ca^{2+}-regulated ABPs, such as profilin and PrABP80. The sustained actin depolymerization may function to maintain a block on tip growth, but preliminary data suggest it may also operate in crosstalk with PCD signalling cascades. The SI-induced PCD cascade includes leakage of cytochrome c from mitochondria, activation of a caspase-like activity and nuclear DNA fragmentation. Thus, the cytoskeleton is not only a target for signalling cascades, but may also be an initiator and transducer of signals. Activation of the p56 MAP kinase occurs after tip growth inhibition and, therefore, may signal to downstream events. Phosphorylation of the soluble inorganic pyrophosphatase, p26, is thought to act as a fail-safe mechanism ensuring that tip growth remains inhibited. It is becoming clear, therefore, that there

is crosstalk between the SI signalling cascades and that PCD probably functions as a decision-making point that results in incompatible pollen being committed to death.

Thus, we are beginning to dissect a complex network of signals and targets involved in signalling cascades triggered by SI. The challenge for the future is not only to identify additional components, but also to elucidate how these components interact and what crosstalk is utilized during SI. In particular, the specific molecular interactions between $[Ca^{2+}]_i$, actin alterations and PCD signalling cascades are currently being investigated. Furthermore, hints that p56 MAP kinase activation may also cross-talk to these elements are being pursued, as there is evidence for MAPK involvement in F-actin alterations and PCD in other systems (Zhang et al. 2000; Yang et al. 2001; Samaj et al. 2002). As the title of this book implies, it appears that plants have some aspects of a neuronal-like system of communication and we are just beginning to scratch the surface of understanding the intricacies of how cellular responses are achieved through networks of signalling intermediates.

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7 Signal Perception and Transduction in Plant Innate Immunity

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Abstract Innate immunity is an ancient form of defense against microbial infection that is shared by plants, insects and vertebrates. Like animals, plants have evolved perception systems for multiple, highly invariant pathogen-associated molecular patterns (PAMP) that trigger basal or non-cultivar-specific defense responses in plants. Non-self-recognition is brought about by specific plant receptors that are structurally similar to Toll-like receptors mediating PAMP perception and activation of innate immune responses in animals. In addition to PAMP-mediated plant innate immunity, disease-resistance programs are often also initiated through plant cultivar-specific recognition of microbial race-specific virulence factors, a recognition specificity that is not known from animals. Plant species and plant cultivar-specific resistance represent evolutionarily linked types of immunity that are collectively referred to as the plant innate immune system. Signal transduction cascades that mediate activation of innate immune responses comprise elements that are common to both forms of plant immunity, such as alterations in cytoplasmic calcium levels, mitogen-activated protein kinase activities or reactive oxygen species production. Forward genetic approaches have, however, identified loci that contribute specifically to either plant species or plant cultivar-specific immunity.

7.1 Introduction

Immunity of multicellular organisms to microbial invasion is based upon the host's ability to discriminate between "self" and potentially dangerous "nonself" structures (Hoebe et al. 2004). Phylogenetically ancient defense mechanisms, termed the innate immune system, make use of nonclonal, germline-encoded pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway 1997). These structures are unique to microbes (not found in potential hosts), are invariant among microorganisms of a given class, and are indispensable for microbial physiology (Medzhitov and Janeway 2002). Innate immunity appears to operate in virtually all higher organisms, including vertebrates, insects and plants (Medzhitov and Janeway 2002; Nürnberger and Brunner 2002). In contrast to this broad phylogenetic distribution of innate immune systems, adaptive immunity arose only recently in evolution and is restricted to jawed vertebrates (Hoebe et al. 2004). This type of immunity is based on somatic recombination mechanisms that generate a large repertoire of antigen receptors which are clonally distributed on T- and B-lymphocytes (Kasahara et al. 2004).

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While plants clearly lack adaptive immunity, their innate immune system consists of two mechanistically and evolutionarily related branches (Nürnberger et al. 2004). PAMP-based activation of innate defense responses is considered to form the molecular basis of plant species-specific or basal resistance, a type of resistance that is effective in all plant cultivars of a given species against all races of a pathogen species (Espinosa and Alfano 2004; Thordal-Christensen 2003). A current model predicts that evolution of pathogen race-specific virulence mechanisms that help pathogens to overcome basal defense responses facilitated the evolution of plant cultivar-specific disease resistance in plants (Espinosa and Alfano 2004). This type of pathogen race/plant cultivar-specific resistance constitutes the second branch of the plant immune system and is governed by the direct or indirect interaction of pathogen race-specific avirulence (Avr) factors and plant cultivar-specific resistance gene products. Thus, PAMPs and Avr factors can clearly be distinguished by their natural distribution (microbial species-specific vs. microbial race-specific) and their plant species or plant cultivar-specific activity profile. In summary, plant species (or plant non-cultivar-specific) and plant cultivar-specific resistance represent a pathogen non-race-specific as well as a pathogen race-specific way of coping with attempted microbial invasion, and should be considered as two distinct, but evolutionarily interrelated types of resistance that constitute plant innate immunity.

7.2 PAMPs as Triggers of Nonplant Cultivar-Specific Innate Immune Responses

PAMP-based activation of innate immunity is almost invariably found in multicellular eukaryotes (Aderem and Ulevitch 2000; Cook et al. 2004; Imler and Hoffmann 2001; McGuinness et al. 2003). Major examples of PAMPs triggering innate immune responses in various vertebrate and nonvertebrate organisms comprise the lipopolysaccharide (LPS) envelope of Gram-negative bacteria, peptidoglycans from Gram-positive bacteria, eubacterial flagellin, methylated bacterial DNA fragments and fungal cell wall derived glucans, chitins, mannans and proteins (Cook et al. 2004; Girardin et al. 2002). Remarkably, many of these molecules act as triggers of immune responses in various plant species as well (Boller 1995; Montesano et al. 2003; Nürnberger et al. 2004; Vorwerk et al. 2004) (Tab. 7.1). Structural conservation of the PAMPs recognized by plants and animals may suggest a common ancestral origin of perception systems in the individual hosts. However, such similarities seem not to extend to the minimum structural requirements for eliciting immunity in both plants and animals.

Table 7.1. Selection of pathogen-associated molecular patterns (*PAMPs*) and of plants sensitive to the respective PAMP

PAMP	Pathogen(s)	Plants sensitive to the respective PAMP	Reference
Lipopolysaccharide	Gram-negative bacteria (xanthomonads, pseudomonads)	Pepper, tobacco, *Arabidopsis*	Meyer et al. (2001), Newman et al. (2002), Zeidler et al. (2004)
Flagellin	Gram-negative bacteria	Tomato, *Arabidopsis*	Felix et al. (1999)
Elongation factor (EF-Tu)	Gram-negative bacteria	*Arabidopsis,* other *Brassicaceae*	Kunze et al. (2004)
Harpin (HrpZ)	Gram-negative bacteria (pseudomonads, Erwinia)	Tobacco, *Arabidopsis*, parsley, cucumber	He et al. (1993), Lee et al. (2001), Wei et al. (1992)
Cold-shock protein	Gram-negative bacteria, Gram-positive bacteria	Tobacco, tomato	Felix and Boller (2003)
Necrosis-inducing proteins	Bacteria (*Bacillus* spp.), fungi (*Fusarium* spp.), oomycetes (*Phytophthora* spp., *Pythium* spp.)	All dicotyledonous plants tested so far	Bailey (1995), Fellbrich et al. (2002), Mattinen et al. (2004), Pemberton and Salmond (2004), Qutob et al. (2002), Veit et al. (2001)
Transglutaminase	Oomycetes (*Phytophthora* spp.)	Parsley, potato	Brunner et al. (2002), Halim et al. (2005), Nürnberger et al. (1994)
Lipid-transfer proteins (elicitins)	Oomycetes (*Phytophthora* spp, *Pythium* spp.)	Tobacco	Osman et al. (2001)
Xylanase	Fungi (*Trichoderma* spp.)	Tobacco, tomato	Enkerli et al. (1999), Ron and Avni (2004)
Invertase	Yeast	Tomato	Basse et al. (1993)
β-Glucans	Fungi (*Pyricularia oryzae*), oomycetes (*Phytophthora* spp.), brown algae	Legumes, tobacco, rice	Fliegmann et al. (2004), Klarzynski et al. (2000), Yamaguchi et al. (2000)
Sulfated fucans	Brown algae	Tobacco	Klarzynski et al. (2003)
Chitin	All fungi	Tomato, *Arabidopsis*, rice, wheat, barley	Baureithel et al. (1994), Ito et al. (1997)
Ergosterol	All fungi	Tomato	Granado et al. (1995)
Cerebrosides A, C	Fungi (*Magnaporthe* spp.)	Rice	Koga et al. (1998)

For example, an N-terminal 22 amino acid fragment of bacterial flagellin is sufficient to activate immune responses in various plants (Felix et al. 1999), but the same domain is dispensable for flagellin perception by animal cells (Donnelly and Steiner 2002). Thus, flagellin recognition may have arisen independently in the two phylae, and likely results from convergent evolution. The evolutionary reason, however, why flagellin perception has arisen twice in plants and animals to activate innate defense systems is likely because flagellin represents a predominant and microbe-specific surface signature that facilitated non-self-recognition.

PAMPs constitute highly conserved determinants typical of whole classes of pathogens that are not found in potential host organisms and that are indispensable for the microbial lifestyle. PAMP-like elicitors of plant immune responses match these characteristics. For example, Pep-13 (Nürnberger et al. 1994), a surface-exposed peptide motif of a *Phytophthora* cell wall transglutaminase (TGase) serves as a recognition determinant for the activation of defense in various plants, such as parsley and potato, in response to infection by various species of the genus *Phytophthora* (Brunner et al. 2002; Halim et al. 2005). Pep-13 sequences are highly conserved among *Phytophthora* TGases, but are not found in plant proteins and the Pep-13 motif is essential not only for elicitor activity but also for TGase activity of the protein. Furthermore, TGase isoforms containing the Pep-13 motif are expressed at all stages of the life cycle of *Phytophthora infestans* including plant infection, suggesting a crucial role of these enzymes in *Phytophthora* biology (Fabritius and Judelson 2003). In a similar study, Felix and Boller (2003) described a cold-shock-inducible RNA-binding protein from various Gram-positive bacteria (RNP-1) that induced innate immune responses in tobacco. Within RNP-1 a central region was defined that was conserved among all RNP-1 orthologs tested from various bacteria. Remarkably, this region was found to be indispensable not only for the RNA-binding activity of the protein but also for its immune-stimulating capacity.

7.3 Plant Pattern Recognition Receptors Mediate PAMP Perception and Activation of Non-Cultivar-Specific Plant Defense

In animals (ranging from crustaceans to insects to vertebrates), PAMP perception is brought about by pattern recognition receptors that distinguish self from conserved microbial structures (Aderem and Ulevitch 2000; Cook et al. 2004; Girardin et al. 2002; Medzhitov and Janeway 2002). *Drosophila* Toll and mammalian Toll-like receptors (TLRs) recognize PAMPs through

an extracellular leucine-rich repeat (LRR) domain and transduce the PAMP signal through a cytoplasmic *Drosophila* Toll/Interleukin-1 receptor (TIR) domain (Cook et al. 2004; Underhill and Ozinsky 2002). Plant binding proteins for numerous microbe-derived compounds have been kinetically and biochemically characterized, but in only a very few cases purification of the receptor protein or isolation of the encoding gene has been achieved (Montesano et al. 2003; Nürnberger et al. 2004). *Fabaceae* species recognize 1,6-β-linked, 1,3-β-branched heptaglucosides (HGs) from the cell walls of the phytopathogenic oomycete, *Phytophthora sojae*, for activation of plant defense through binding to a 75-kDa HG-binding protein (HGP) (Mithöfer et al. 1999). Intriguingly, soybean HGP harbors endoglucanase activity that releases oligomeric 1,3-β-D-oligoglucosides consisting of at least four moieties from complex glucans (Fliegmann et al. 2004). Thus, upon contact with *Phytophthora*, HGP may facilitate release of oligoglucoside fragments from the oomycete cell wall that are appropriate PAMPs for binding to the glucan-binding domain of the same protein. As HGP does not show any transmembrane domain it is likely to interact in concert with other proteins in PAMP perception and intracellular signal transduction. Remarkably, PAMP perception systems in animals are often multicomponent complexes as well (Underhill and Ozinsky 2002).

The flagellin receptor, FLS2, from *Arabidopsis thaliana* constitutes a transmembrane receptor protein kinase with an extracellular LRR domain (LRR-receptor-like kinase, LRR-RLK) (Gomez-Gomez and Boller 2000). Extracellular LRR domains are also found in the *Drosophila* Toll receptor and in the ten vertebrate TLRs (Cook et al. 2004; Underhill and Ozinsky 2002), all of which are implicated in the activation of innate immune responses. Little sequence similarity between the LRR domains of FLS2 and the animal flagellin receptor, TLR5, and the aforementioned fact that both receptors recognize different structures of eubacterial flagellin make it very likely that both receptors evolved independently as a result of convergent evolution (Gomez-Gomez and Boller 2000; Hayashi et al. 2001). This is even more evident as FLS2 and TLR5 also differ in the structure of their cytoplasmic domains. While FLS2 harbors a cytoplasmic Ser/Thr protein kinase domain (Gomez-Gomez and Boller 2000), TLR5 carries an intracellular TIR domain that is indirectly associated with the interleukin-1-receptor associated kinase (IRAK) via the adaptor protein MyD88 (Hayashi et al. 2001). It is remarkable, however, that LRR domains have evolved independently for extracellular ligand recognition, which likely reflects the unique features of this domain for mediating intermolecular interactions. In that respect it is worth noting that the plant brassinosteroid receptor, BRI1, directly binds the brassinolide (a plant steroid hormone) through one of the LRR stretches (Kinoshita et al. 2005). This suggests that LRR domains may not only facilitate protein–protein interactions, but may even act as

pattern recognition modules that universally mediate perception of ligands of all chemical kinds. As plants possess 235 LRR-RLKs (Shiu and Bleecker 2001), a significant number of those proteins are expected to serve PAMP perception in plants.

Loss of flagellin perception in *Arabidopsis* resulted in enhanced disease susceptibility (reduced basal resistance) against the virulent bacterial strain, *Pseudomonas syringae* pv. *tomato* DC3000 (Zipfel et al. 2004). This is important as it indicates that PAMP perception actively contributes to basal (or plant species-specific) immunity and that single PAMP recognition events already affect the severity of microbial infections on susceptible host plants. Eventually, this efficiency may explain why suppression of PAMP-mediated immunity evolved as a major strategy of microorganisms to establish susceptibility on host plants.

7.4 Pathogen Recognition in Host Cultivar-Specific Resistance

A current model suggests that PAMP-induced plant species (nonhost) resistance was incapacitated by microbial pathogens through the acquisition of virulence factors which enabled them to colonize susceptible hosts through suppression of innate immune defenses (Espinosa and Alfano 2004). In turn, evolution of new pathogen race-specific virulence factors have driven the coevolution of plant cultivar-specific resistance genes and thus development of phylogenetically more recent pathogen race/plant cultivar-specific disease resistance (Dangl and Jones 2001; Van der Hoorn et al. 2002). The genetic basis for plant cultivar-specific disease resistance is determined by gene pairs called pathogen-derived avirulence (*Avr*) genes and plant-derived resistance (*R*) genes. *Avr* gene-encoded proteins are likely (sometimes dispensable) effectors that contribute to host infection, although their biochemical mode of action in many cases remains elusive. In those cases when Avr factors are recognized by resistant host plant cultivars through interaction with their complementary *R* gene-encoded protein counterparts, they act as specific elicitors of plant defense rather than virulence or pathogenicity factors and betray the potential phytopathogen to the host surveillance system.

A simple biochemical interpretation of this gene-for-gene hypothesis would be a receptor/ligand-like interaction between plant *R* gene products and the corresponding pathogen-derived *Avr* gene products. Direct interaction between AVR proteins and R proteins was indeed shown (Cohn et al. 2001; Jia et al. 2000), but nevertheless represents an exception rather than the rule in *R*-gene-dependent plant immunity. Molecular analyses of numerous plant–microbe interactions revealed that the situation is likely

much more complex and that R proteins are unlikely to act as receptors for microbe-derived Avr proteins (Dangl and Jones 2001; Jones and Takemoto 2004). In many cases, binding sites for Avr proteins have been found not only in resistant but also in susceptible plant cultivars, suggesting that those sites serve as virulence targets for microbial effectors during attempted infection (Axtell and Staskawicz 2003; Dangl and Jones 2001; Mackey et al. 2002). These findings form the basis of the so-called guard hypothesis, which predicts that AVR proteins act as virulence factors that contact their cognate pathogenicity targets in host plants or even nonhost plants, but only function as elicitors of cultivar-specific plant resistance when the complementary R protein is recruited into a functional signal perception complex. Thus, R proteins monitor ("guard") AVR-mediated perturbance of cellular functions and may thus be considered adapter proteins that – owing to their presence – initiate signaling cascades that are suppressed in susceptible plant cultivars that lack the appropriate R protein. A prime example constitutes the *Arabidopsis RPM1* gene that confers resistance against *P. syringae* strains expressing the type III effectors, AvrRpm1 and AvrB. RPM1 "guards" the plant against pathogens that manipulate RIN4 (the pathogenicity target in the plant) via AvrRpm1 or AvrB (bacterial virulence factors) in order to suppress host defenses (Mackey et al. 2002). RIN4 also appears to be the target for another *P. syringae* pv. *tomato* derived AVR protein, AvrRpt2 (Axtell and Staskawicz 2003). However, in contrast to the previously described situation AvrRpt2 does not assemble with RIN4 and RPM1, but with RIN4 and its cognate R protein, RPS2, which confers resistance against bacterial strains expressing AvrRpt2, but not AvrRpm1 or AvrB, respectively. RIN4 likely acts as a negative regulator of RPS2-mediated resistance in the uninfected plant. Upon infection, plant cyclophilin-assisted autocatalytic activation of the cysteine protease, AvrRpt2, resulted in AvrRpt2-mediated breakdown of RIN4 and subsequent activation of RPS2-mediated plant immunity (Coaker et al. 2005).

Intracellular plant R proteins fall into one of two major structural classes. Motifs commonly found in R proteins of one class are coiled-coil (CC, leucine zipper) domains, LRRs and a nucleotide-binding site (CC-NBS-LRR) (Jones and Takemoto 2004). A second, widely found subset of plant *R* genes comprises a TIR domain in conjunction with a NBS and an LRR domain (TIR-NBS-LRR) (Jones and Takemoto 2004). Plant plasma membrane R proteins are either composed of extracellular LRR domains only or are fused to protein kinase domains (LRR-RLK), such as in the *Xa21* gene from rice (Song et al. 1995). Notably, the domain structure of Xa21 resembles that of the plant PAMP receptor, FLS2.

Importantly, similar structures are typical for the architecture of PAMP perception modules in animal cells as well (McGuinness et al. 2003; Medzhitov and Janeway 2002; Underhill and Ozinsky 2002). For example,

intracellular NBS-LRR proteins carrying an additional CARD domain (NOD1, NOD2) are implicated in intracellular PAMP sensing in animals (Girardin et al. 2002), while TIR-LRR proteins are implicated in PAMP sensing at the animal cell plasma membrane (Underhill and Ozinsky 2002). Thus, structural modules implicated in PAMP perception in animals do not only resemble plant PAMP perception systems (FLS2 vs. TLR5), but also perception systems for pathogen race-specific Avr proteins.

7.5 Intracellular Signal Transduction in Plant Innate Immunity

Signaling pathways mediating PAMP-induced defense responses employ altered cytoplasmic Ca^{2+} levels, reactive oxygen species (ROS), nitric oxide (NO) and posttranslationally regulated mitogen-activated protein kinase (MAPK) activity (Jonak et al. 2002; Nürnberger et al. 2004; Zhang and Klessig 2001). Intriguingly, most of these components are important for PAMP-induced activation of innate immune responses in animal cells (Barton and Medzhitov 2003). NO production appears to be a common characteristic of plants that are under pathogen attack (Clarke et al. 2000; Delledonne et al. 1998; Durner et al. 1998). Although plant enzymes that are homologous to human NO synthase (hNOS) have not been reported, pharmacological evidence has been presented for a plant enzyme that mechanistically resembles the human ortholog (Delledonne et al. 1998; Durner et al. 1998). Recently, Zeidler et al. (2004) showed that AtNOS1, a plant-specific NOS previously associated with hormone signaling in plants (Guo et al. 2003), mediated LPS-induced NO production and *PR* gene expression in *A. thaliana* (Zeidler et al. 2004). Importantly, inactivation of the AtNOS1 gene did not only abrogate LPS-induced NO production in these plants, but also dramatically enhanced susceptibility of the mutant to *P. syringae* pv. *tomato* DC3000 infection.

MAPKs constitute central points of crosstalk in stress signaling, including those that result in protection against microbial invasion (Gomez-Gomez and Boller 2002; Jonak et al. 2002; Nürnberger and Scheel 2001; Zhang and Klessig 2001). Transient posttranslational activation of MAPK activity has been reported from various pathogen-infected plants or from plants that were infiltrated with different PAMPs. In particular, two isoforms of a total of 20 *Arabidopsis* MAPKs (AtMPK3 and AtMPK6) and their homologous proteins in other plant species are responsive to PAMP treatment or pathogen infection (Jonak et al. 2002; Zhang and Klessig 2001). In PAMP-treated parsley cells, PcMPK3 and PcMPK6 translocate to the nucleus (Lee et al. 2004; Ligterink et al. 1997), where they likely contribute to ROS-independent, WRKY transcription factor-dependent *PR* gene expression

(Eulgem et al. 1999; Kroj et al. 2003). Moreover, Asai et al. (2002) identified a flagellin-induced MAPK cascade and WRKY transcription factors that act downstream of FLS2, and described a role of MAPK in activating early defense gene transcription likely through WRKY transcription factor activity. Although transcription factors are likely to be phosphorylation substrates of plant MAPKs, the only proteins known to be directly phosphorylated by AtMPK6 are two isoforms of 1-aminocyclopropane-1-carboxylic acid synthase (ACS), the rate-limiting enzyme of ethylene biosynthesis (Liu and Zhang 2004). MPK6-catalyzed phosphorylation of ACS2 and ACS6 results in accumulation of ACS protein, elevated levels of cellular ACS activity, ethylene production and ethylene-induced plant defense phenotypes. Further functional links between MAPK activation and the plant immune response, that is *PR* gene expression and the initiation of programmed cell death, were established in *Arabidopsis* and tobacco, respectively (Jonak et al. 2002; Menke et al. 2004; Nürnberger et al. 2004; Ren et al. 2002; Zhang and Klessig 2001). For example, one study provided evidence that species immunity in *Nicotiana benthamiana* was crucially dependent on NbSIPK and NbWIPK (orthologous to AtMPK6 and AtMPK3). Virus-induced gene silencing of either of the two isoforms resulted in significant reduction of transcript levels that allowed massive growth of *P. cichorii*, which would not multiply on wild-type *N. benthamiana*. Intriguingly, silencing of the heat-shock proteins HSP70 and HSP90 in the same system also compromised nonhost resistance to *P. cichorii*, suggesting a crucial role of chaperone activity in this particular type of plant resistance (Kanzaki et al. 2003). As NbHSP90 was shown to interact with NbSIPK in a yeast two-hybrid system, it is conceivable that chaperones may constitute scaffolds that stabilize plant MAPK cascades. A requirement for chaperone activity (a particular HSP90 isoform of *Arabidopsis*) has recently been demonstrated also for *R*-gene-dependent resistance (Takahashi et al. 2003).

Forward and reverse genetics approaches have contributed substantially to our current understanding of the molecular requirements of plant immunity. The analysis of mutants impaired in hormone homeostasis revealed that jasmonic acid, ethylene and salicylic acid are not only crucial to cultivar-specific host plant resistance, but may also be indispensable for the maintenance of nonhost resistance in specific plant–microbe combinations (Mysore and Ryu 2004). NPR1, a protein that modulates expression of *PR* genes in a salicylic acid dependent manner, was recently shown to be regulated by its redox status which facilitated complex formation with transcription factors in the nucleus and subsequent binding to *PR* gene promoters and *PR* gene expression (Mou et al. 2003). EDS1 and PAD4 are lipase-like proteins that were previously shown to be important for cultivar-specific host resistance governed by TIR-NBS-LRR genes, while NDR1, a protein of unknown function is essential for cultivar-specific host

resistance mediated by CC-NBS-LRR resistance genes (Dangl and Jones 2001; Jones and Takemoto 2004). SGT1 and RAR1, both components of the plant Skp1–Cullin–F-box (SCF) protein ubiquitin ligase complex (supposedly implicated in the removal of negative regulator plant resistance pathways), have been implicated in innate immunity both at the plant species and the plant cultivar level (Azevedo et al. 2002; Peart et al. 2002; Takahashi et al. 2003).

7.6 Conclusions and Future Prospects

The last decade has seen an enormous increase in the knowledge base on plant innate immunity. Cloning and characterization of more than 50 plant resistance genes and isolation of the first genes encoding plant PAMP receptors are among those achievements. Establishment and proof of the "guard hypothesis" and elucidation of the role of microbial effector proteins in susceptible (virulence factors) and resistant host plant cultivars (Avr factors) has further helped us to reconcile the concepts of PAMP-based immunity and *R* gene-dependent cultivar-specific plant immunity. Importantly, conceptual and structural similarities in the molecular organization of plant and animal innate immunity have been unraveled. While these findings underline the requirement for non-self-recognition and rejection in all multicellular eukaryotic organisms, the currently available data set supports independent, convergent evolution of innate immunity in plants and animals.

In the future, a combination of powerful genetic screens and reverse genetics approaches, protein–protein interaction studies and genomic analyses (the latter based upon an increasing number of fully sequenced microbial genomes, including those from closely related pathovars) will significantly enhance the number of elements implicated in plant innate immunity. A more complete picture of the molecular basis of the two faces of this phenomenon (plant species and plant cultivar-specific innate immunity) will enable us to determine the genetic requirements that are common or specific to either type of plant immunity. Moreover, understanding the molecular organization of durable plant species immunity is likely to open avenues towards genetic engineering of durable pathogen resistance in crop plants.

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8 Nitric Oxide Involvement in Incompatible Plant–Pathogen Interactions

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Abstract Most plants resist potential parasite attack using a variety of biochemical responses that often lead to a localized cell death termed the hypersensitive response, and include production of antimicrobial compounds, lignin formation, oxidative and nitrosative burst, and increased expression of genes related to pathogenesis. In this framework, nitric oxide (NO) functions together with reactive oxygen species in triggering hypersensitive cell death, and works independently of such intermediates in the induction of defense-related genes. In this chapter, we will examine the synthesis of NO and its signaling functions in the hypersensitive response and in the establishment of systemic acquired resistance.

8.1 Introduction

Nitrogen monoxide or nitric oxide (NO) is a bioactive molecule that exerts a number of diverse activities in phylogenetically distant species (Beligni and Lamattina 2001). It is a gaseous free radical with a relatively short half-life, estimated to be less than 6 s (Bethke et al. 2004). This short half-life reflects the highly reactive nature of NO. Its broad chemistry involves an interplay between three species differing in their physical properties and chemical reactivity: the nitrosonium cation (NO^+), the radical ($NO^\bullet$) and the nitroxyl anion (NO^-) (Neill et al. 2003). Typically, NO rapidly reacts with reactive oxygen species (ROS), proteins, especially with reactive amino acids such as cysteine and tyrosine, as well as with various receptors and transcription factors (Romero-Puertas et al. 2004). NO was first described in mammals as a major messenger in neurotransmission and is also involved in vascular smooth muscle relaxation and regulation of vasoprotection. Macrophages and other circulating cells produce NO, which mediates their bactericidal and tumoricidal effects (Delledonne et al. 2003). Research on NO in plants has gained considerable attention in recent years, and there is increasing evidence of a role of this molecule in plant growth, development and defense (Romero-Puertas et al. 2004).

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8.2 Activation of the Defense Response

Plants exhibit a wide array of both passive and active defense strategies against pathogen attack. Preformed physical barriers (e.g., the cuticle and the cell wall) and biochemical defenses (e.g., antimicrobial toxins) are often insufficient to avoid spread of infection. Therefore, after recognition of the invading pathogen, plants activate a very effective arsenal of inducible defense responses (hypersensitive response, HR) characterized by hypersensitive cell death, tissue reinforcement and production of antimicrobial metabolites (McDowell and Woffenden 2003; Dangl et al. 1996; Hammond-Kosack and Jones 1996). The rapid recognition of pathogen infection is supported by a sophisticated surveillance system that is capable of distinguishing between self-generated signals and those emitted by pathogens (Holub 2001). It is followed by the establishment of systemic acquired resistance (SAR), a salicylic acid dependent long-lasting immunity against a broad spectrum of pathogens (Ryals et al. 1996). The HR requires active host protein synthesis and is kept under tight genetic control, being activated only if the plant detects a potential invader (Baker et al. 1997). In this way, plant cells autonomously maintain constant vigilance against pathogens by expressing large arrays of "*R* genes" (R, resistance). *R* genes encode putative receptors that respond to the product of "*Avr* genes" (Avr, avirulence) expressed by a pathogen during infection (McDowell and Woffenden 2003). This gene-for-gene interaction results from either direct or indirect interaction between the *R* gene and *Avr* gene products depending on the *R*–*Avr* gene pair. Evidence is emerging that R proteins often do not recognize Avr proteins directly. In the "guard hypothesis," R proteins activate resistance by interacting with another plant protein that is modified by the pathogen (McDowell and Woffenden 2003).

R-gene-mediated activation of HR triggers a number of rapid cellular responses, including perturbations in ion fluxes and the pattern of protein phosphorylation, which precede the accumulation of ROS (mainly O_2^- and H_2O_2) and NO as well as the transcriptional activation of defense-related genes (McDowell and Dangl 2000; Cohn et al. 2001) (Fig. 8.1). These initial responses are followed by the production of phytoalexins, transcriptional activation of defense genes and hypersensitive cell death, which is a form of programmed cell death having regulatory and mechanistic features that are similar to apoptosis in animal cells, such as membrane dysfunction, cytoplasmic vacuolization, chromatin condensation and endonucleolytic cleavage of DNA (Gilchrist 1998). The interplay between ROS and NO contributes to the establishment of the HR and to the potentiation of defense responses (Blume et al. 2000). Downstream events are inhibited by some

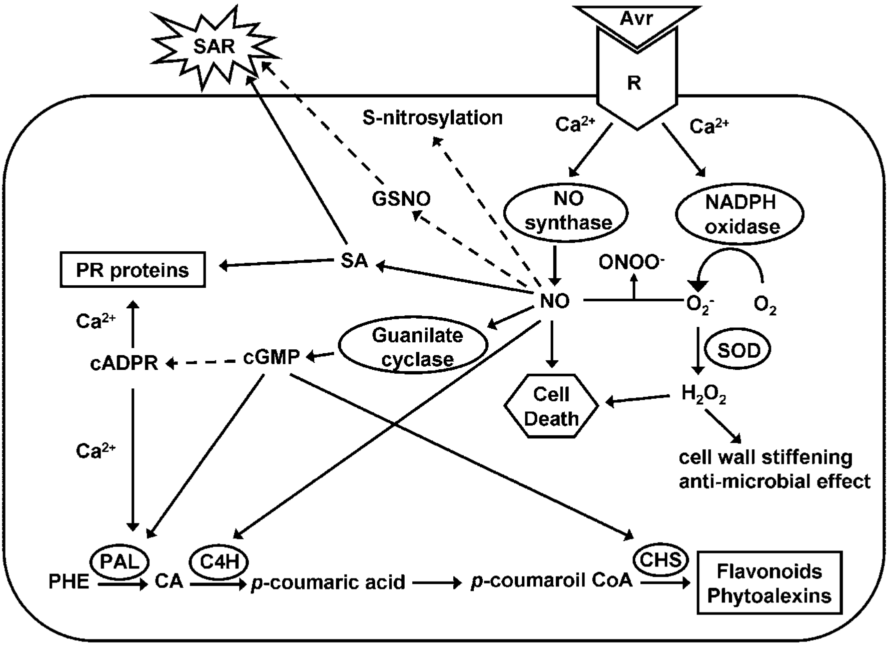

Fig. 8.1. Representation of NO signaling functions during the hypersensitive response. *Dashed lines* represent potential NO functions; *solid lines* represent experimental supported results. *Avr* avirulence signal, *CHS* chalcone synthase, *C4H* cinnamic acid-4-hydroxylase, *CA* cinnamic acid, Ca^{2+} calcium influx, *cADPR* cyclic ADP ribose, *cGMP* cyclic GMP, *GSNO* *S*-nitroso-l-glutathione, *PAL* phenylalanine ammonia lyase, *PHE* phenylalanine, *PR proteins* pathogenesis-related proteins, *R* receptor, *SA* salicylic acid, *SAR* systemic acquired resistance, *SOD* superoxide dismutase

blockers for Ca^{2+} channels and anion channels, suggesting that the initial ion fluxes are crucial for the induction of defense responses (Wendehenne et al. 2002).

8.3 NO Production During the Hypersensitive Disease Resistance Response

In mammals, biosynthesis of NO is primarily catalyzed by NO synthase (NOS) by oxidation of l-arginine to l-citrulline and NO. In plants, NO can be produced through both nonenzymatic and enzymatic routes. The former include the light-mediated conversion of NO_2 to NO catalyzed by carotenoids (Cooney et al. 1994). Liberation of NO occurs from nitrite at neutral pH (Yamasaki and Sakihama 2000). Other nonenzymatic reactions occur at acidic pH and in the presence of reducing agents such as

ascorbate and phenolic compounds (Romero-Puertas et al. 2004). Recently, the synthesis of NO via nonenzymatic reduction of apoplastic nitrite in seeds has been demonstrated (Bethke et al. 2004), although it is unlikely that this route of production of NO is significant in response to pathogen attack as the apoplastic pH is likely to be too high (Bolwell et al. 2002). The enzymatic routes of NO synthesis include reduction of nitrite by nitrate reductase (NR), conversion of nitrite by xanthine oxidoreductase (Rockel et al. 2002) and conversion of arginine to citrulline by NOS (Guo et al. 2003). As NR is involved in many physiological responses (Neill et al. 2003), no significant differences in NO accumulation have been observed in response to infection with avirulent pathogens between wild-type *Arabidopsis* plants and a mutant impaired in NR activity (Zhang et al. 2003). Recently, AtNOS1 from *Arabidopsis thaliana* was identified on the basis of its sequence similarity to a protein implicated in NO synthesis in the snail *Helix pomatia* (Guo et al. 2003). AtNOS1 displays flavin-, heme- and tetrahydrobiopterin-independent NOS activity and appears to be constitutively expressed (Zeidler et al. 2004).

In plants, NO production in response to infection has been shown under conditions in which the generation of ROS is also activated (Delledonne et al. 1998; Clarke et al. 2000; Foissner et al. 2000; Allan and Fluhr 1997). This event is concomitant with the avirulent gene-dependent oxidative burst that occurs immediately prior to the onset of hypersensitive cell death (Romero-Puertas et al. 2004). In *A. thaliana* and soybean, NO production rapidly increases following challenge with avirulent bacteria and is maintained over a 6-h period (Clarke et al. 2000; Delledonne et al. 1998; Zhang et al. 2003). Moreover, direct contact of avirulent crown rust fungus with oat plants induces the production of NO in an early stage in the defense response (Tada et al. 2004). In addition, epidermal tobacco cells treated with the fungal elicitor cryptogein (Foissner et al. 2000; Lamotte et al. 2004) and potato tubers treated with an elicitor from *Phytophthora infestans* (Yamamoto et al. 2004) accumulate NO within minutes. Lipopolysaccharides extracted from Gram-negative plant and animal pathogens have also been found to elicit a strong and rapid burst of NO in *A. thaliana* plants (Zeidler et al. 2004).

8.4 Experimental Approaches for Manipulation of Endogenous NO Levels

Most of the current information about the function of NO in plants relies on data obtained by pharmacological studies. These approaches include application of NO donors or NO scavengers, and loss of NO synthesis

through inactivation of NO-generating enzymes (Neill et al. 2003). Various NO donors have been employed to elevate the level of NO in plant cells. Although some compounds release NO, others are either iron nitrosyl substances with a strong NO^+ character or release equimolar amounts of NO and superoxide anion (O_2^-) and should therefore be considered as sources of peroxynitrite ($ONOO^-$). As a consequence, data obtained using these molecules are often difficult to interpret. Recently, it has been shown that donors releasing NO in different redox forms have different or even opposite effects on the expression of ferritin (Murgia et al. 2004). Sodium nitroprusside, a NO donor that releases NO^+, induces the accumulation of ferritin transcripts in *A. thaliana* cell suspensions. In contrast, other NO donors such as *S*-nitroso-*N*-acetylpenicillamine and *S*-nitroso-L-glutathione (GSNO) were not able to induce such an accumulation (Murgia et al. 2004). The NO scavengers 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) and carboxyPTIO are commonly used to reduce the levels of NO (Neill et al. 2003). Mammalian NOS inhibitors are another effective approach widely used in modulating NO levels in plants (Neill et al. 2003). Recently, additional genetic-based approaches to manipulate NO levels in plants in order to clarify the NO involvement in incompatible plant–pathogen interaction have been proposed (Zeier et al. 2004). *A. thaliana* AtNOS1 mutant plants were found to be more susceptible to virulent *Pseudomonas syringae*: these plants displayed a much severer development of disease symptoms and enhanced bacterial growth compared with wild-type (Zeidler et al. 2004). Furthermore, the expression in *A. thaliana* plants as well as in avirulent *P. syringae* of genes encoding bacterial flavohemoglobins, which possess a strong NO denitrosylase activity, revealed that the removal of NO from either the inside or the outside causes a reduction in hypersensitive cell death (Zeier et al. 2004). Similarly, tobacco plants that overproduce a nonsymbiontic hemoglobin from alfalfa, which can act as a NO scavenger, exhibited reduced cell death after inoculation with avirulent pathogens (Seregelyes et al. 2003) although this protection was not observed in *A. thaliana* that express a nonsymbiotic hemoglobin challenged with an avirulent strain of *P. syringae* (Perazzolli et al. 2004).

8.5 NO and Cell Death

In animal cells, NO has been shown to cooperate with ROS to induce DNA fragmentation and cell lysis in murine lymphoma cells, hepatoma cells and endothelial cells. In this case, cell death is characterized by chromatin condensation, vacuolization of the cytoplasm and loss of the mitochondrial membrane electrical potential. Accumulating evidence also indicates a role

of NO in plant hypersensitive cell death. In *A. thaliana* suspension cells, NO donors induce cell death at concentrations similar to those generated by cells challenged by avirulent bacteria (Clarke et al. 2000). In soybean cell suspensions, cell death appears to be mediated by relative levels of NO and H_2O_2 (Delledonne et al. 2001). The same results were seen in tobacco cultured cells where the addition of a NO donor and a H_2O_2 generator separately had no effect on cell viability. However, when NO and H_2O_2 were added simultaneously, cells died in a concentration-dependent manner (de Pinto et al. 2003). Whereas the mechanism by which NO and H_2O_2 interact for killing is still largely unknown, the reaction between NO and O_2^- produces $ONOO^-$ (Koppenol et al. 1992), a highly reactive oxidant molecule that interacts with many molecular components and may modulate downstream signaling (Beckman et al. 1990). In mammals, $ONOO^-$ induces apoptosis and is also cytotoxic by causing oxidative tissue damage (Lin et al. 1995). In plants $ONOO^-$ does not appear to be an essential intermediate of NO-induced cell death (Delledonne et al. 2001). However, $ONOO^-$ induces *PR-1* accumulation in tobacco leaves (Durner and Klessig 1999) and protein nitration leading to changes in the redox state of the cell (Delledonne et al. 2001).

8.6 NO Signaling in the Plant Defense Response

The mobile nature of NO and its chemical reactivity with various cellular targets means that the downstream effects of NO may be directly induced by interaction of NO with various cellular components such as ion channels or proteins that modulate gene expression, or indirectly following interaction of NO with signaling proteins (Neill et al. 2003). In mammalian systems, one of the most important targets of NO is guanylate cyclase (GC). NO interacts with the heme prosthetic group of this enzyme, leading to its activation and resulting in an increased generation of intracellular cyclic GMP (cGMP). In plants, a similar activation of GC has also been suggested (Hancock et al. 2002). Administration of NO donors or recombinant mammalian NOS to tobacco plants or suspension cells triggers the expression of *PR-1* and phenylalanine ammonia lyase (PAL) (Durner and Klessig 1999). These genes are also induced by cGMP, suggesting that the NO/cGMP-dependent signaling pathway present in animals may also exist in plants. In addiction, two inhibitors of NO-inducible GC are able to suppress induction of *PAL* in tobacco plants, suggesting the involvement of cGMP-dependent components in NO-dependent defense gene activation. However GC inhibitors produced only a partial suppression of *PAL* transcripts, suggesting the existence of both cGMP-dependent and cGMP-independent pathways (Durner et al. 1998).

In both animal and plant cells, cyclic ADP ribose (cADPR) is another molecule that serves as a second messenger for NO signaling. In both systems, cADPR functions as a second messenger to stimulate Ca^{2+} release through intracellular Ca^{2+}-permeable ryanodine receptor channels. cADPR was shown to induce *PAL* and *PR-1* gene expression in tobacco, and this induction was blocked by a cADPR-gated Ca^{2+} channel inhibitor (Durner and Klessig 1999). Moreover, a cADPR antagonist such as 8-Br-cADPR partially suppressed NO-dependent induction of *PR-1* transcripts, suggesting that NO activation of the defense response may occur through more than one pathway (Delledonne et al. 2003). Since the expression of *PR-1* and *PAL* genes was increased when cGMP and cADPR were added simultaneously, these two second messengers appear to act synergistically to increase gene expression. Recently, Lamotte et al. have shown in tobacco cells that NO participates in the cryptogein-mediated elevation of cytosolic free Ca^{2+} through the mobilization of Ca^{2+} from internal stores. In addition, cryptogein-mediated cytosolic Ca^{2+} elevation was shown to be partially inhibited at low concentrations of an antagonist of RYR channels (Lamotte et al. 2004). These results suggest that NO contributes to the release of Ca^{2+} from internal pools.

It is now recognized that NO and its related species can introduce post-translational modification of proteins. These modifications are due to the high reactivity of NO with the thiol groups present in reactive amino acids such as cysteine and tyrosine as well as the transition metal centers of a wide functional spectrum of proteins (Stamler et al. 2001). Tyrosine nitration and methionine oxidation can introduce irreversibile modification of proteins, leading to loss of function, while cysteine nitrosylation is a reversibile modification that can modulate protein functions (Sokolovski and Blatt 2004). These modifications are both reversible and specific, allowing cells to flexibly and precisely alter protein function in response to environmental signals (Mannick and Schonhoff 2002).

8.7 Systemic Acquired Resistance and NO

SAR is activated after pathogen infection and leads to the induction of the plant defense response in uninfected parts of the plant. As a result, the entire plant is more resistant to a secondary infection. Salicylic acid plays an important role during incompatible interactions for the amplifications of early signals deriving from plant-pathogen recognition (Shirasu et al. 1997). Exogenous application of salicylic acid has been found to mimic SAR and induces the transcription of *PR* genes. Several lines of evidence highlight the role of NO in the modulation of signaling leading to SAR,

although its activity is fully dependent on the functions of salicylic acid. In tobacco, NO treatment has been shown to result in the accumulation of salicylic acid and its conjugates (Durner et al. 1998). *PR-1* induction by NO is mediated by salicylic acid as it is blocked in NahG transgenic plants that are unable to accumulate salicylic acid (Durner et al. 1998). Moreover, treatment of tobacco plants with NOS inhibitors or NO scavengers partially inhibits salicylic acid induced SAR (Song and Goodman 2001).

Although salicylic acid moves through the plant, it is not an essential signal that activates SAR (Mauch-Mani and Métraux 1998). Several molecules, such as short peptides and selected lipids and lipid derivatives, have been suggested to be putative short- or long-distance signals mediating the development of a variety of defense mechanisms. Yet another candidate for mobile signals is GSNO (Durner and Klessig 1999). GSNO is a major metabolite in phloem that is distributed throughout the plant and is believed to act as both an intracellular and an intercellular NO carrier. In mammals NO has been shown to react with glutathione GSH to form GSNO, which can serve as a systemic source of NO. In addition, GSNO has been shown to induce systemic resistance against tobacco mosaic virus infection (Song and Goodman 2001), and *PAL* expression in tobacco (Durner et al. 1998). A GSNO-catabolizing enzyme (glutathione dependent formaldehyde dehydrogenase, GS-FDH) and its encoding gene have recently been characterized. Mutant yeasts that lack this gene showed enhanced susceptibility to nitrosative challenge, indicating an important biological role for this enzyme. The identification of the GS-FDH gene both in pea and *Arabidopsis* suggests the ability of the plant to modulate GSNO bioactivity and signaling functions. (Neill et al. 2003).

8.8 Conclusions and Future Prospects

NO is a gas with a broad chemistry that involves an array of interrelated redox forms with different chemical reactivities. NO was named "Molecule of the Year" in 1992 by the journal *Science,* a Nitric Oxide Society was founded, and a scientific journal devoted entirely to NO was created. A number of recent publications have evidenced the broad spectrum of cellular functions modulated by NO in plants. NO takes part in the regulation of several physiological processes, but the molecular mechanisms by which NO operates are still largely unknown. A great effort is now needed for the identification and characterization of the direct targets of NO. The understanding of NO signaling functions at the biochemical, cellular and molecular levels will soon make it possible to discern several important physiological and pathological processes of plants, as has already been demonstrated in mammals.

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9 From Cell Division to Organ Shape: Nitric Oxide Is Involved in Auxin-Mediated Root Development

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Abstract Roots are plant organs that mainly function to acquire water and nutrients from soil. Root development is under the control of a regulated cell proliferation and morphogenesis, and auxin is the central plant hormone that governs those processes. In this review we discuss new aspects of the mechanisms that operate during root organogenesis. We particularly emphasize the analyses of downstream signals involved in the auxin control of root development. Nitric oxide (NO), an emerging chemical messenger that plays a significant role in a broad spectrum of plant developmental processes, is a key component in the signal transduction pathways that determine root architecture. Lateral root development as well as adventitious root formation are strictly NO-dependent processes in the auxin-promoted root organogenesis.

9.1 Introduction

Roots are organs that accomplish a variety of biological functions. They are the site of nutrient and water uptake and they also constitute the storage organ in some species. Roots are in contact with a complex environment including soil microorganisms in the rhizosphere and are, thereby, submitted to a fine modulation and cross talk of information, signals and stimuli. Roots are able to produce and to sense growth regulators, chemical messengers and metabolites that communicate to the whole plant the result of processing and integration of that information. In this review we present recent data concerning the downstream signals involved in auxin-regulated mechanisms that control root growth and development. We focus on the involvement of nitric oxide (NO) as a signal molecule leading to lateral and adventitious root development with the aim of integrating available data and to generate a clearer scenario for describing the cross talk between auxins and NO.

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9.1.1
Auxins Control Root Development

Hormones are essential players for transmitting information and connecting the whole plant. The action of hormones involves its perception, initiation of both specific and nonspecific responses and, finally, the result of a new steady state of growth and/or metabolic conditions. Among hormones, auxin plays an important role during plant growth and developmental processes; therefore, one of the essential aims of plant biologists is to understand its action, regulation and target molecules. As a critical plant hormone, auxin modulates diverse processes such as tropic responses to light and gravity, root and shoot morphogenesis, organ patterning, vascular development and growth in tissue culture (Davies 1995). Auxin is known to influence cell division, cell expansion and cell differentiation and to have a profound influence on root morphology. The control of root length, the enhancement of lateral root (LR) formation and root hair density, and the induction of adventitious root development are widely known auxin-induced processes. Mutants that overproduce auxins tend to have abundant lateral and adventitious roots (Boerjan et al. 1995; King et al. 1995); conversely, mutants deficient in auxin responses are often characterized by long primary roots and few LRs (Estelle and Somerville 1987; Hobbie and Estelle 1995).

Although many tissues can synthesize auxin (Ljung et al. 2001), it is mainly produced in the shoot apical meristem. It was postulated that the rate and direction of auxin movement through the cells and finally through the whole plant is the key fate in auxin-mediated responses (Leyser 1998). Auxin transport is complex and highly regulated, involving many identified proteins (Morris 2000). One of the most characteristic features of auxin is its polar cell-to-cell transport (Jones 1998). Both acropetal (Wilkins and Scott 1968) and basipetal (Davies and Mitchell 1972) transport occurs in roots. Opposing directions of auxin transport in roots is achieved by spatial separation. Chemical and genetic approaches have revealed that transport of auxin to distant sites is clearly required for normal development (Friml 2003). For example, indole acetic acid (IAA, the main active auxin in plants) transport is necessary for proper LR development (Reed et al. 1998; Bhalerao et al. 2002; Casimiro et al. 2001). Recent research suggests that polar transport of auxin is accomplished via vesicular secretion linked to endocytotic and recycling processes (Baluska et al. 2003). This would imply that in addition to the hormone-like and morphogen-like properties, auxin could also have a neurotransmitter-like behavior (Baluska et al. 2003, 2004). IAA biosynthesis, metabolism and transport are key features to orchestrate plant development. Moreover, mechanisms downstream of auxin transport must necessarily transduce the auxin message and be relevant

to complete its biological function. In spite of molecular, genetic and biochemical information generated through more than 100 years of studies on auxin biological functions, the auxin-controlled signaling pathways during root development as well as the specific roles for intermediate molecules involved are not fully understood.

9.1.2 Nitric Oxide Is a New Player in Auxin-Mediated Root Development: Summary of Its Effects

NO is a diffusible second messenger first described in mammals, where it plays variable functions ranging from dilation of blood vessels to neurotransmission and defense during immune response (Gow and Ischiropoulos 2001). Numerous investigations in the last decade have discovered new functions for this molecule in the plant kingdom (Lamattina el al. 2003; Neill et al. 2003). NO affects in a noticeable manner the morphology and developmental pattern of roots. NO was shown to be involved in the promotion of lateral (Correa-Aragunde et al. 2004) and adventitious (Pagnussat et al. 2002) roots. High concentrations of NO produce root growth inhibition (Correa-Aragunde et al. 2004), while low concentrations induce root elongation (Gouvea et al. 1997; Hu et al. 2005). The density of root hairs is increased by exogenous application of NO in various plant species (Lombardo and Lamattina, unpublished results). Recently, it was also demonstrated that NO mediates the gravitropic bending in soybean roots (Hu et al. 2005). Figure 9.1 shows that NO effects on plant root morphology and physiology take place at many different locations along the root organ. Interestingly, all these NO-mediated effects in roots are also under the control of auxins. Moreover, local NO accumulation occurs in response to auxins during adventitious and LR emergence and also in the gravitropic response of roots (Pagnussat et al. 2002; Correa-Aragunde et al. 2004; Hu et al. 2005).

Various NO sources have been identified in plants and many of them have been described in roots. Enzymatic sources that include nitrate reductase and NO synthase like activities are involved in NO production (Rockel et al. 2002; Guo et al. 2003). In parallel, apoplastic synthesis of NO from nitrite at an acidic pH might also be relevant (Bethke et al. 2004). Roots are regularly exposed to nitrite, and acidification of root apoplast often occurs in association with altered nutrient supply. Interestingly, cell wall and apoplast acidification can also be induced by auxin (discussed by Bethke et al. 2004). Additionally, a plasma membrane nitrite reductase specific of roots has been described to catalyze the formation of NO from nitrite (Stohr and Ulrich 2002). Finally, the potential NO source derived

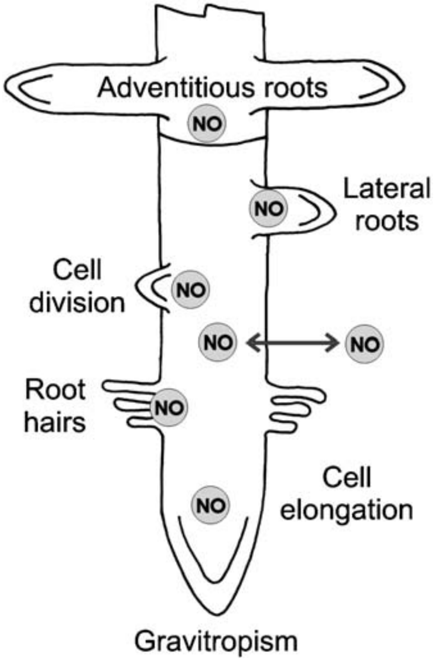

Fig. 9.1. Schematic model showing nitric oxide (*NO*) participation in different processes during root growth and morphogenesis. NO generated endogenously or applied exogenously has been implicated in signaling pathways associated with root physiology and development: (1) adventitious root formation from parenchyma cells at the root–shoot junction (Pagnussat et al. 2002); (2) lateral root development from differentiated pericycle cells (Correa-Aragunde et al. 2004); (3) root hair induction (Lombardo and Lamattina, unpublished results); (4) root cell elongation, stimulation at low NO concentrations (Gouvea et al. 1997) and inhibition at high ones (Correa-Aragunde et al. 2004); (5) cell division, NO positively regulates cell cycle-promoting genes during lateral root primordia formation (Correa-Aragunde et al., submitted); (6) gravitropic response (Hu et al. 2005)

from the activity of the microorganisms living in the rhizosphere, among them the plant growth-promoting bacteria, must also be considered (Creus et al. 2005).

In this context, it becomes evident that NO could be synthesized in roots and that its synthesis depends on root environment and internal conditions. Evidence suggests that auxin could be an important factor in determining NO production in roots. Here we summarize the tight linkage between auxins and NO signaling during root development, particularly during lateral and adventitious root formation (ARF).

9.2 Nitric Oxide Mediates Auxin-Induced Lateral Root Development

LRs play a major role in taking up nutrients and water from soil and strongly contribute to the physical support for the plant. Therefore, the ability of plants to develop a branched root architecture greatly increases their success in a particular environment (Malamy and Benfey 1997a). LRs originate from differentiated nondividing pericycle cells within the primary root. The first event during LR primordia formation occurs when individual cells from the pericycle are induced to dedifferentiate and divide symmetrically and asymmetrically to form the LR primordium. Finally, the LR primordium grows principally by elongation and emerges from the parent root (Malamy and Benfey 1997a). The initiation of LRs is dramatically influenced by information derived from a wide range of environmental, genetic and physiological factors (Malamy and Benfey 1997b; Casimiro et al. 2003). Hence, the plant must integrate these signals and decide whether or not to trigger LR initiation in a specific zone of the primary root. Thereby, several interesting questions arise: (1) how are these cues perceived and interpreted?, (2) how are the signals transduced and (3) how is the localized organogenesis initiated? The identification of the nature of these signals and the understanding of how they interact to regulate LR development are important challenges for plant biologists.

Auxin has been known for a long time to be the main plant hormone involved in LR development. It was recently shown that NO is required for auxin-mediated LR formation (Correa-Aragunde et al. 2004). The application of the NO donor sodium nitroprusside induces LR development in tomato (*Lycopersicon esculentum* L.) seedlings, while specific scavenging of NO results in no LR formation (Fig. 9.2). The NO effect is dose-dependent, displaying a typical hormone dose–response curve. Moreover, NO is able to induce LR primordia in auxin-depleted seedlings and it was found that auxin-induced LR formation could be prevented by application of the NO scavenger 2-(4-carboxyphenyl)-4, 4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (CPTIO) (Correa-Aragunde et al. 2004). These results strongly support a lineal signal transduction cascade involving NO downstream of auxins. NO is mainly produced in the pericycle cells that will give place to an LR, indicating that NO is required during early stages of LR development. According to this, depletion of endogenous NO with CPTIO results in the complete inhibition of LR primordia formation in the CPTIO-treated seedlings. In parallel to the promotion of LR formation, it was clearly demonstrated an NO dose-dependent inhibition of primary root growth (Correa-Aragunde et al. 2004; Fig. 9.2). Microscopical analysis

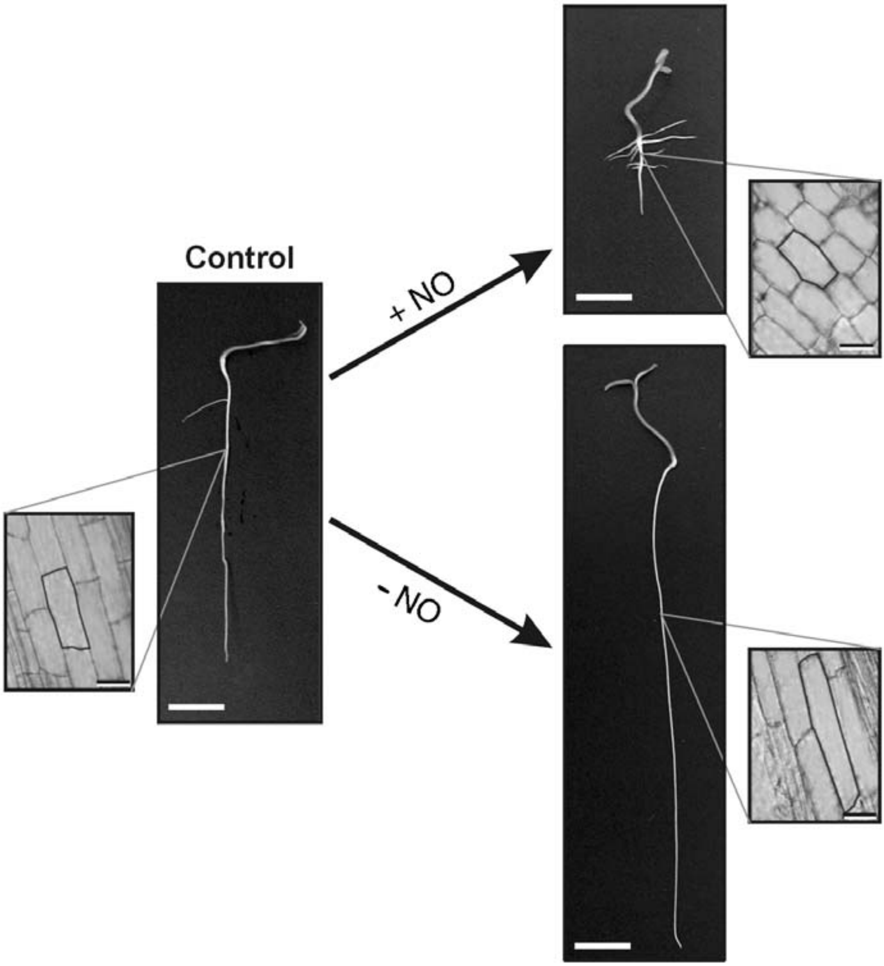

Fig. 9.2. NO modulates root architecture. Photographs of tomato seedlings incubated in water (*control*), 1 mM of the NO scavenger 2-(4-carboxyphenyl)-4, 4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt (*–NO*) and 200 μM of the NO donor sodium nitroprusside (*+NO*) for 5 days. *Bar*: 1 cm. *Insets* Representative photographs of fully elongated primary root cells. *Bar*: 50 μm

showed that the NO-dependent reduction in primary root length correlates with an inhibition in root cell elongation (Fig. 9.2, inset).

LRs originate from a zone distal to the actively dividing primary root meristem. Hence, during the initiation of LRs, pericycle cells must dedifferentiate and reenter the cell cycle. The plant hormone auxin promotes the G1-to-S phase transition through the expression of cell cycle regulatory genes like cyclins and cyclin-dependent kinases (CDK) in some pericycle

cells named founder cells. Moreover, auxin negatively regulates the expression of two CDK inhibitors: Kip-related protein 1 (KRP1) and 2 (KRP2), involved in the inhibition of G1-to-S phase transition in *Arabidopsis* (Himanen et al. 2002). Interestingly, NO was able to induce G1-to-S phase transition through the expression of cyclins and the downregulation of KRP2 during LR formation in tomato (Correa-Aragunde et al., submitted).

Results clearly show that NO is involved in the control of LR initiation and development. Whether or not NO is the signal that integrates environmental, physiological and genetic factors that conduce to LR formation and hence modifies root architecture is a yet unanswered question. It remains to be explored if any factor that stimulates LR development could also induce NO production in roots. For example, nutrients such as nitrate have an important impact on LR development. In soils or media with patchy nitrate distribution, LRs preferentially proliferate in the nitrate-rich zone (Granato and Raper 1989; Zhang and Forde 2000). Since enzymatic and nonenzymatic nitrate and nitrite reduction could produce NO in roots (Rockel et al. 2002; Stohr and Ullrich 2002; Bethke et al. 2004), an interesting point to analyze is whether NO could also be the signal that announces the presence of nitrate in the soil and triggers the formation of LRs in the nutrient-rich zone. Additionally, the nitrification and denitrification reactions produced by soil microorganisms are probably the most important NO sources in the soil. Thus, the NO released by microorganisms could have a significant impact on root architecture. Recently, it has been reported that the rhizobacterium *Azospirillum brasilense* produces NO and that it is involved in the *Azospirillum*-mediated LR promotion in tomato (Creus et al. 2005).

9.3 Nitric Oxide Is Required for Adventitious Root Formation

Auxin is the hormone responsible for the induction of ARF, a process that involves cell division and root primordia development. Auxin promotes dedifferentiation of parenchyma cells and the entrance to cell division to form a new root meristem (De Klerk et al. 1995; Fujita and Syôno 1996).

A few years ago it was demonstrated that NO is involved in the auxin response during ARF in cucumber (Pagnussat et al. 2002). A transient increase in endogenous NO concentration after IAA treatment was reported in the basal region of the hypocotyl, where the new root meristems develop (Pagnussat et al. 2002). More recently, it was proposed that convergent and complex cyclic GMP (cGMP) dependent and independent signaling pathways are orchestrating the formation of a new root system when the primary root is removed (Pagnussat et al. 2003, 2004).

9.3.1
Nitric Oxide Acts Downstream of Auxins to Induce Adventitious Root Formation

The auxin IAA is synthesized in the shoot apical meristem of the seedlings and is basipetally transported via the polar transport system (Jones 1998). When the apical IAA production was disrupted by decapitation of cucumber explants and basipetal transport of auxins was inhibited by treatment with 1-naphthylphthalamic acid, ARF was significantly reduced (Pagnussat et al. 2003). Interestingly, this inhibitory effect due to blockage of auxin production and transport could be reversed by NO, suggesting that IAA and NO might be acting through a lineal signaling pathway. Accordingly, the auxin-induced ARF was prevented by the application of the NO scavenger CPTIO. Altogether, these results indicate that NO operates downstream of auxins triggering ARF.

9.3.2
Nitric Oxide Activates Cyclic GMP Dependent Pathways During Adventitious Root Formation

In mammalian systems, one of the most studied targets of NO is the enzyme guanylate cyclase (GC). GC, together with the enzyme phosphodiesterase (PDE), regulates the endogenous concentration of the cellular messenger cGMP. NO is able to transiently activate GC and increase the level of cGMP, while PDE activity is responsible for the breakdown of cGMP into 5′ GMP. cGMP is an important signaling molecule involved in mechanisms that sense extracellular stimuli and transduce the signals into metabolic responses (Reggiani 1997). The involvement of cGMP in different plant physiological processes has been assessed by the use of inhibitors of the enzyme GC like 6-anilino-5, 8-quinilinedione (LY83583; Mulsch et al. 1988; Donaldson et al. 2004). The GC inhibitor LY83583 was able to reduce ARF in both IAA- and NO-treated cucumber explants. This inhibition was reversed by the treatment with the permeable cGMP analog 8-Br-cGMP. In addition, the PDE inhibitor sildenafil citrate mimicked the NO effect on ARF (Pagnussat et al. 2003). This evidence strongly indicates that NO operates downstream of IAA inducing ARF through the GC-catalyzed synthesis of cGMP.

A potential target for cGMP is a cGMP-dependent protein kinase (Pk-G). Although no plant Pk-G has been cloned yet, biochemical evidence for a Pk-G activity has recently been reported in the plant morning glory (Szmidt-Jaworska et al. 2003). cGMP can also act via cyclic ADP ribose (cADPR). cADPR was reported to regulate cytosolic Ca^{2+} concentration in various plant systems (Sanders et al. 1999). Consequently, variations in Ca^{2+}

concentration may be involved in the signaling events leading to ARF in cucumber. Results obtained in our laboratory indicate that Ca^{2+} and Ca^{2+}-dependent protein kinase (CDPK) activity are downstream messengers in the signaling pathways triggered by auxins and NO. Moreover, the availability of both intracellular and extracellular Ca^{2+} pools seems to be required for the action of IAA and NO to trigger ARF (Lanteri et al., submitted). Thus, in addition to the function of Ca^{2+} as a mineral nutrient modulating the root growth (Druart 1997; Bellamine et al. 1998), evidence supports the involvement of Ca^{2+} as a second messenger linking both auxins and NO to the activation of processes leading to ARF (Fig. 9.3).

9.3.3 Nitric Oxide Induces Cyclic GMP Independent Pathways During Adventitious Root Formation

Diverse signal transduction cascades rely on mitogen-activated protein kinases (MAPKs) as intermediates to regulate a variety of cellular functions in response to extracellular stimuli. Experimental evidence from different plant species indicates that several MAPK pathways are implicated in the regulation of cell cycle and developmental processes, and in responses to environmental constraints and hormone treatments (Tena et al. 2001; Jonak et al. 2002). Evidence supports the activation of a MAPK signaling cascade occuring in response to auxins during ARF. Interestingly, this activation was shown to be mediated by NO. The MAPK cascade seems to be cGMP-independent, since the NO-induced in vitro MAPK activity was not affected by the GC inhibitor LY83583. However, the NO-induced MAPK activity dramatically decreased when measured in the presence of the MAPK kinase inhibitor PD098059 (Pagnussat et al. 2004).

The MAPK signaling pathway could be regulating both mitotic processes and the expression of auxin-induced genes during the formation of new roots. Convincing evidence on the requirement of a MAPK cascade for plant cell division comes from experiments carried out in tobacco and *Arabidopsis* cell cultures. It has been shown that the expression of the tobacco MAPK kinase kinase (MAPKKK) NPK1 (nucleus and phragmoplast-localized protein kinase 1) is essential for phragmoplast expansion, and its absence results in the formation of multinucleate cells (Nishihama et al. 2001). In addition, Krysan et al. (2002) showed that members of the *Arabidopsis* NPK-like protein kinase family are involved in the control of cell division in *Arabidopsis*. The induction of MAPK activity in response to auxins had previously been described by Mizoguchi et al. (1994) and Mockaitis and Howell (2000). Interestingly, studies of tobacco have revealed that NO activates a MAPK involved in the plant defense responses (Kumar and Klessig 2000).

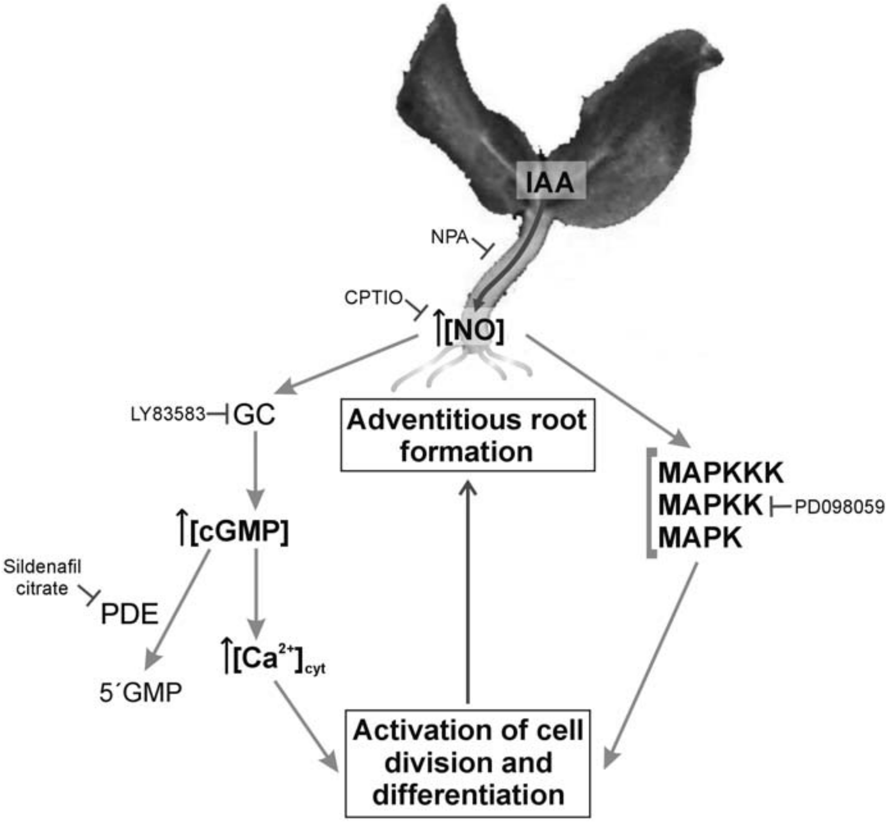

Fig. 9.3. Schematic representation that integrates the signaling pathways and molecules involved in the indole acetic acid (*IAA*) – and the NO-induced adventitious root formation (*ARF*) in cucumber explants. The auxin IAA is synthesized in the shoot apical meristem of the seedling and is basipetally transported to the basal region of the hypocotyl. There, IAA triggers a local and transient generation of NO in a yet unknown manner (Pagnussat et al. 2002). Thereafter, an NO-mediated cyclic GMP (*cGMP*) dependent pathway that probably includes the modulation of cytosolic Ca^{2+} concentration is operative to trigger ARF (Pagnussat et al. 2003; Lanteri et al., submitted). In parallel, a cGMP-independent pathway that involves the activation of a mitogen-activated protein kinase signaling cascade is required for ARF (Pagnussat et al. 2004). Both pathways could mediate downstream responses including the induction of cell division and differentiation resulting in ARF. *NPA* 1-naphthylphthalamic acid, *GC* guanylate cyclase, *PDE* phosphodiesterase, ⊥ inhibition

Overall, the available data suggest a picture in which basipetal transport of auxins induces an NO burst in the basal region of the hypocotyl, where the adventitious root primordia develop. Then, NO triggers a bifurcated signaling cascade that includes both cGMP-dependent and -independent pathways. The activation of both pathways seems to be required for ARF

in cucumber explants since the effect of both auxins and NO is abolished when one of them is compromised (Fig. 9.3).

9.4 Conclusions and Future Perspectives

We are clearly taking the first steps in order to describe at what different levels NO modulates root morphology and physiology. Nevertheless, the major challenge will be the integration of the myriad of signals involved in root development and the linkage between them and the different enzymatic and nonenzymatic NO sources in roots.

Considerable effort and imagination will be necessary to design innovative approaches and experimental models which should include different factors affecting the signals associated with root growth. The availability of water, oxygen and nutrients, the interaction with soil microorganisms (pathogens and nonpathogens), the production and modification of plant growth-promoting compounds by those organisms together with the genetic background of each plant species should be considered and integrated in the near future. The application of genetic tools and the analysis of root growth behavior under different soil conditions will surely contribute to deciphering the participation of root systems in plant productivity. In turn, NO which has been postulated as a key player in many of the known auxin-mediated processes associated with root development, should be considered as a mammalian-like neurotransmitter when describing its behavior in future discussions.

The basic knowledge discussed in this review will clearly have a tremendous impact owing to its potential application for improving programs of rooting that are actually being used in nurseries. Undoubtedly, NO actions should be taken into account when studying the specific requirements of each plant species that determines its root growth pattern.

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10 Neurotransmitters, Neuroregulators and Neurotoxins in Plants

Susan J. Murch

Abstract The transmission of signals between living cells is essential for the life of an organism as it provides the mechanism by which cells respond to external stimuli. Plants produce a wide range of phytochemicals that mediate cell function and translate environmental cues for survival and many of these are human neuroregulatory molecules. For example, the human neurotransmitter melatonin (*N*-acetyl-5-methoxytryptamine) is a ubiquitous, highly conserved molecule associated with timing of circadian rhythms in many organisms, including higher plants. Other compounds such as hyperforin, now isolated from several plant species, may function as serotonin transport or nonspecific cation channel activators in human brains and potentially in higher plants. A different group of neuroregulatory molecules produced by plants overstimulate human neurons, resulting in neuronal cell damage and death. Excitotoxins such as β-methylamino-L-alanine not only affect human health but are also regulatory molecules redirecting plant growth. Many fascinating questions in future research will be defining the role of neurotransmitters, neuroregulators and neurotoxins in the growth and development of plants. As newer technologies emerge, it will become possible to understand more about the role of neurological compounds in the inner workings of plant metabolism, plant environment interactions and the impact of plant neurosystems on human neurology.

10.1 Neurotransmitters: Signaling Molecule in Plants?

Neurotransmitters found in plants to date include acetylcholine, epinephrine, dopamine, levodopa, γ-aminobutyrate (GABA), glutamate, indole-3-acetic acid, 5-hydroxyindole acetic acid, melatonin and serotonin (Fig. 10.1). The human "fight or flight" neurohormones, catecholamines, promote flowering in short day plants (Khurana et al. 1987) and accumulate when plants are stressed (Swiedrych et al. 2004). Other human neurohormones, such as GABA, function as mechanisms to conserve nutrients under stress conditions in plants or as antiherbivory compounds (Shelp et al. 2003). Another class of human neurohormones, indoleamines, can also influence plant growth and development (Murch and Saxena 2002a). Melatonin (*N*-acetyl-5-methoxytryptamine) was first isolated from the bovine pineal gland in 1958 (Lerner et al. 1958). In the human brain, the highest levels of melatonin are found during deep sleep and melatonin levels decline with sunrise. The amplitude of the fluctuation of melatonin concentration declines with age, a phenomenon associated with sleep rhythm disorders,

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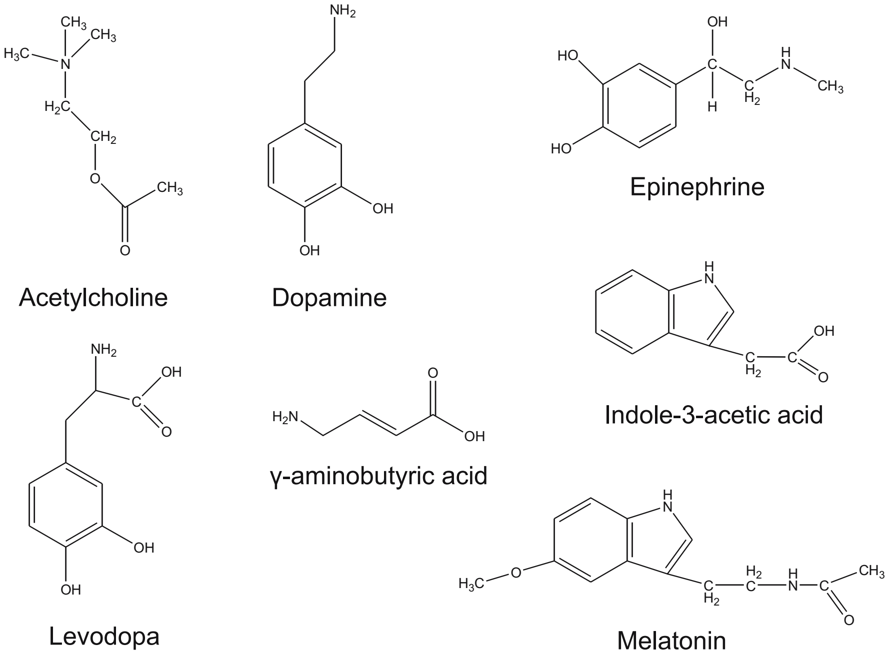


Fig. 10.1. Neurotransmitters found in a wide range of plants

geriatric insomnia, jet lag, epilepsy, seasonal affective disorder, pain, gastric ulcers, cancer, body temperature irregularities, migraine and depression (Yu and Reiter 1993). The function of reproductive systems in photoperiodically dependent rodents is largely controlled by the release of melatonin from the pineal gland (Wurtman et al. 1963; Reiter 1991). In addition, melatonin is an effective free-radical scavenger and antioxidant (Reiter et al. 1997).

Melatonin was first described in nutritional studies of fruits and vegetables to determine if ingested melatonin could play a role in human health (Dubbels et al. 1995; Hattori et al. 1995). Low quantities of plant melatonin were found in foodstuffs and melatonin is absorbed and active in animal model systems (Hattori et al. 1995). In 1997, significantly higher concentrations of melatonin were detected in medicinal plants with traditional and anecdotal evidence of efficacy as treatments for human neurological ailments (Murch et al. 1997). The discovery of melatonin in medicinal plants was interesting from several perspectives. It was the first report of melatonin in growing higher plants. The neurotransmitter was present in a broad range of plant species from different genera, different countries and different types of ecosystems. Neurotransmitters could impact both plant and human physiology. More recent studies have shown melatonin in

high levels in 108 plant species used in traditional Chinese medicine (Chen et al. 2003). The fundamental questions underlying the discovery of melatonin continue to be centered on the role of melatonin in the metabolism of higher plants. It has been hypothesized that the role of melatonin in plants may be analogous to its function in mammals as a chemical messenger of light and dark, calmodulin binding factor or an antioxidant (Balzer and Hardeland 1996).

To investigate these questions, in vitro cultures of several medicinal plants were established to ensure that the quantified melatonin did not originate from a bacterial or fungal contaminant. Melatonin has been quantified in axenic cultures of *Hypericum perforatum*, *Arabidopsis thaliana*, *Scutellaria baicalensis*, *Artemisia judaica* and many others (Murch et al. 1977; Murch and Saxena, unpublished results). The recovery of radiolabel from ^{14}C-tryptophan as ^{14}C-melatonin and ^{14}C-serotonin in sterile St. John's wort plantlets provided the first evidence of endogenous biosynthesis (Murch et al. 2000). Circadian rhythms in melatonin concentration were observed in *Chenopodium rubrum* (Kolar et al. 1997) and melatonin concentrations were highest at specific stages of flower bud development (Murch and Saxena 2002b). In *A. thaliana*, melatonin levels were significantly higher in sterile etiolated seedlings than in those seedlings that had been grown in light (Fig. 10.2). Additionally, significantly higher levels of melatonin were found in mature ripe tomatoes than in green tomatoes (Van Tassel et al. 2001) and melatonin concentrations varied from 2 to 190 ng g in the seeds of

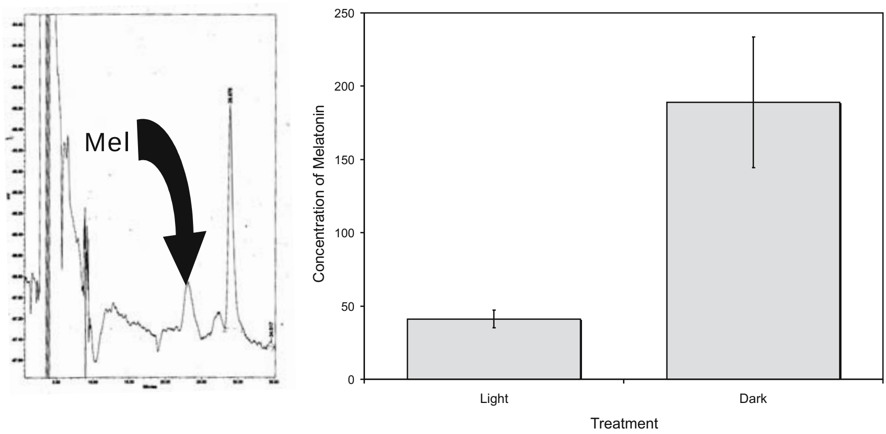


Fig. 10.2. Melatonin detection and quantification in *Arabidopsis thaliana*. **a** Melatonin was detected by high-performance liquid chromatography separation with electrochemical detection as described previously (Murch et al. 2000). **b** Significantly more melatonin was found in sterile etiolated seedlings germinated and grown in complete darkness than was quantified in seedlings grown with a 16-h photoperiod

edible plants (Manchester et al. 2000). Exogenous application of melatonin to plant tissues was shown to increase the birefringence and number of mitotic spindles in lily cells (Jackson 1969) and to disrupt mitosis in onion root cells (Banerjee and Margulis 1973). Melatonin also promoted vegetative growth of etiolated hypocotyls in lupins (*Lupinus albus*) (Hernandez-Ruiz et al. 2004). Exogenously applied melatonin also affected the early steps in the transition to flowering, thereby reducing the number of flowers initiated in *Chenopodium rubrum* (Kolar et al. 2003). These results indicate an association between melatonin and photoregulated metabolic processes in plants such as flowering, seed and root development and is indicative of how much there is left to discover about the inner mechanisms of plant life, plant signaling and plant behavior. However, signaling molecules, by their nature, are short-lived, unstable, difficult to detect and difficult to quantify.

To further investigate the role of melatonin in plant metabolism, a series of inhibitors of the melatonin synthesis pathway were evaluated. There are a wide range of compounds commercially available that mediate the serotonin–melatonin pathway as these are common pharmaceutical targets for medications for depression, migraine and attention-deficit disorders. Four inhibitors of auxin and indoleamine metabolism in plants were identified from a group of more than 20 pharmaceutical compounds by exposure of axenic cultures and quantification of auxin, serotonin, melatonin and related metabolites (Murch 2000). *p*-Chlorophenylalanine, D-amphetamine, fluoxetine (Prozac) and methylphenidate (Ritalin) were shown to alter the rooting potential of stem and hypocotyl explants of St. John's wort (Murch et al. 2001, 2004). In general, significant reductions in de novo root regeneration were found to correspond with decreases in the pool of both indole acetic acid and melatonin. In one instance, root organogenesis was impaired when melatonin concentration decreased but auxin concentration remained unchanged, indicating that the compounds are interdependent (Murch et al. 2001). Interestingly, *p*-chlorophenylalanine significantly decreased the endogenous concentration of melatonin to below detection limits with concurrent increases in serotonin concentration. *p*-Chlorophenylalanine is produced as a pharmaceutical compound that depletes mammalian serum serotonin concentrations (Yamada et al. 1999). In plants, *p*-chlorophenylalanine mediated the same the biochemical pathway and was manifested as a morphological response characterized by the total inhibition of root organogenesis and the development of shoots (Fig. 10.3; Murch et al. 2001).

In 1957, Skoog and Miller hypothesized that the redirection of plant growth is initiated by changes in the relative ratio of plant growth regulators, viz., auxins and cytokinins. Auxin, often refereed to as "master controller" (Trewavas 1997), is among the best characterized metabolites of tryptophan (Fig. 10.4). Auxins induce a polarized growth, while cytokinins

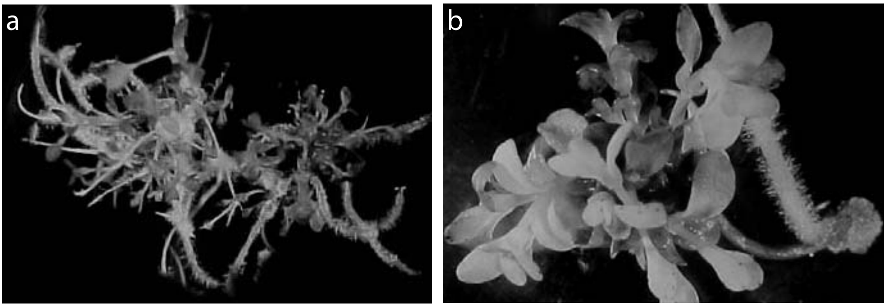


Fig. 10.3. Effect of the melatonin synthesis inhibitor *p*-chlorophenylalanine on morphogenesis in St. John's wort: **a** control explant, **b** explant cultured on medium supplemented with *p*-chlorophenylalanine. Quantification of indoleamines in St. John's wort tissues exposed to *p*-chlorophenylalanine demonstrated the complete inhibition of melatonin synthesis and elevated concentrations of serotonin

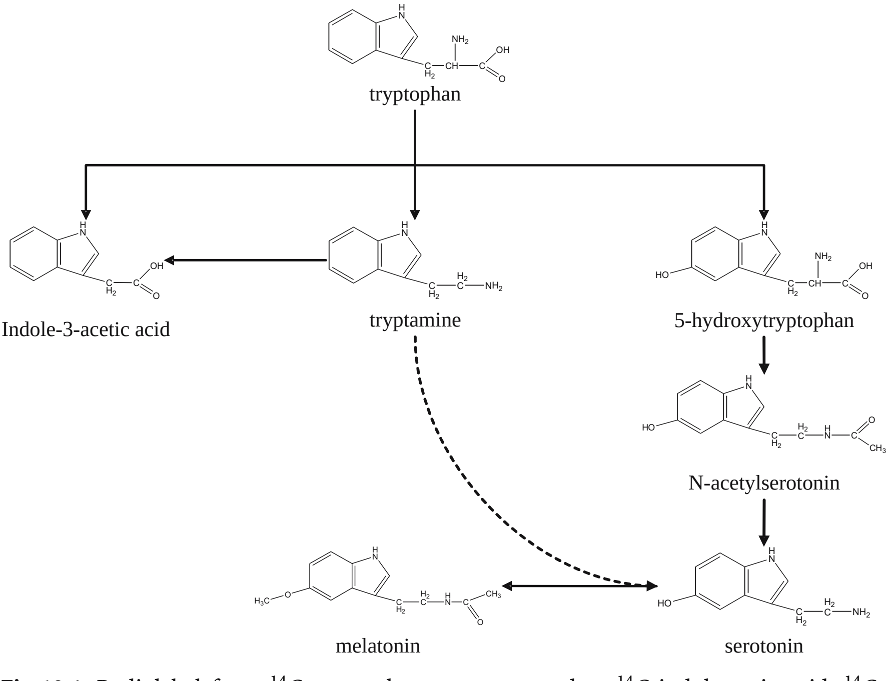


Fig. 10.4. Radiolabel from ^{14}C-tryptophan was recovered as ^{14}C-indoleacetic acid, ^{14}C-tryptamine, ^{14}C-serotonin and ^{14}C-melatonin. (Adapted from Murch et al. 2000, 2002)

attenuate auxin-generated growth. A high ratio of auxin to cytokinin will encourage the regeneration of long, thin tissues such as adventitious roots, whereas a low auxin-to-cytokinin ratio ensures the regeneration of the thicker, more isodiametric tissue forms that are associated with shoots.

However, over the last 45 years, there have been numerous reports of other compounds that modulate the auxin:cytokinin responses or of synthetic compounds that induce regenerative responses in the absence of exogenous auxin or cytokinin. As well, one of the problems inherent in attributing plant responses to the exogenous application of a plant growth regulator may be the failure to incorporate the metabolism of the molecule into the model. Therefore, the various plant responses to auxin may actually represent the responses of plant tissue to a range of auxin-derived or auxin-stimulated metabolites. An alternate potential role for melatonin may be in the photoregulation of plant morphogenic processes, specifically those associated with auxin. A classic bioassay of auxin activity in etiolated lupin hypocotyls indicated that melatonin can play an auxenic role in plant physiology (Hernandez-Ruiz et al. 2004). Further reexamination of the classic systems of plant physiology may provide new clues to the role of melatonin that may be independent of other growth regulators, may mediate other growth regulators or may be involved in the expression of responses attributed to other compounds.

10.2 Neuroregulators in Plants

Plants are among the most diverse of the biological kingdoms and the sheer number of species provides for an almost endless potential for discovery. Plants are also limited by an inability to flee from hostile environments and have evolved a series of strategies for survival that include secondary metabolism for the synthesis of numerous interesting molecules. Many of these molecules mediate neurological processes and therefore are used as pain medications, hallucinogens, antidepressants and poisons. It has been estimated that plant species produce more than 35,000 unique biochemicals that are not required for normal growth or development. Since insects lack a blood brain barrier, neurologically targeted plant compounds can have an immediate and often disruptive or fatal effect. The production of secondary metabolites that are neuroregulating compounds provides plants with a defense system against predators as these compounds affect receptors as agonists or antagonists or they modulate various steps in neuronal signal transduction such as ion channels or enzymes that take up or degrade neurotransmitters.

One of the essential shared experiences in virtually every human culture is a gathering to consume a plant preparation that alters mood, experience or feeling. We meet for coffee (*Coffea arabica*), share a cup of tea (*Camellia sinensis*), or gather to smoke khat (*Celastrus edulis*) or a cigarette (*Nicotiana tabacum*). Traditional cultures in many parts of the world also use

hallucinogenic plants such as Iboga (*Tabernathe iboga*), Yopo (*Andenanthera perigrina*), Caapi (*Banisteriopsis caapi*), Angel's trumpet (*Brugmansia* sp.), Peyote (*Lophorora williamsii*) and Ebena (*Virola theiodora*). Still others exploit the capacity for manipulation of brain metabolism by plants such as marijuana (*Cannabis sativa*), morphine (*Papaver somniferum*) and cocaine (*Erythroxylon coca*).

The examination of even the commonest medicinal plants can reveal surprising chemical diversity. The efficacy of St. John's wort (*Hypericum perforatum* L.) for treatment of human ailments has a long history, perhaps beginning with the description of the plant by the Greek physician Hippocrates in the fifth century bce but one of the greatest challenges in St. John's wort research has been the failure to discover a single molecule to account for the various medicinal efficacies. There have been 193 medicinal metabolites reported in the literature on St. John's wort (*Hypericum perforatum* L.) and compiled in the NAPRALERT database (Murch et al., unpublished results). Hyperforin (Fig. 10.5) is thought to be involved in the antidepressant activity of St. John's wort through inhibition of reuptake of several neurotransmitters and activation of nonselective cation channels (Treiber et al. 2005). In vitro studies have shown that hyperforin interacts with neurotransmitter transporter proteins and reduces the accumulation

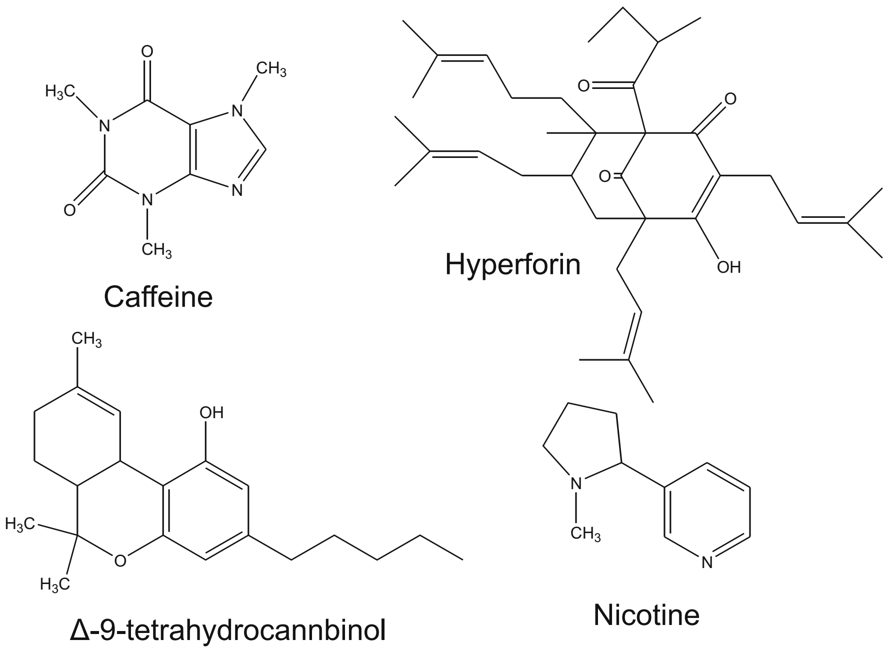


Fig. 10.5. Commonly used neuroregulatory molecules from plants

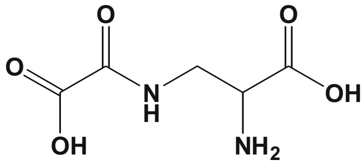
of monoamine, amino acid and acetylcholine in synapses (Chatterjee et al. 1998; Buchholzer et al. 2002). Evidence that hyperforin modulates mammalian neurotransmitter metabolism has also been found in in vivo studies (Buchholzer et al. 2002). Interestingly, hyperforin is not unique to St. John's wort but was recently also found in a completely different medicinal plant species, *Scutellaria baicalensis* Georgi. (Murch et al. 2004b). Therefore, a role for hyperforin in plant metabolism seems likely.

The ability to detect, quantify and optimize production of neuroregulatory molecules from plants is crucial to the discovery of new molecules and mechanisms for treatment of human diseases. Plant-derived cannabinoids have a variety of medicinal properties, including analgesia, antiemesis, antiglaucoma via reduction of intraocular pressure, and reduction of injury (Biegon 2004). Plant symptoms of multiple sclerosis and mediation of the repercussions of traumatic brain-based neuroprotective compounds are especially useful since the surgical repair of neurological damage is frequently impossible. Neuroprotective agents from plants include antioxidants, NO synthase inhibitors, AMPA antagonists, Ca^{2+} channel blockers, estrogen agonists and others (Levi and Brimble 2004).

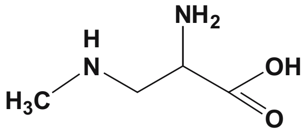
In a recent study, we discovered 781 medicinal compounds in *S. baicalensis*, including 27 antibiotics, and new amino derivatives of baicalin and baicalein that were not previously known (Murch et al. 2004b). The concentration and chemical profile of medicinal species was found to be dependent on both the germplasm (Murch et al. 2004b) and the growth environment (Murch et al. 2003; Zobayed et al. 2003). Indeed, St. John's wort plantlets exposed to a metal ion stress completely lost the capacity to produce hyperforin or hypericin (Murch et al. 2003); therefore, the neurological activity of a whole plant preparation could vary with the preparation of plant tissues from different regions and different seed collections. As well, the often-identified marker compounds thought to be characteristic of a single species may not be unique and may not be related to the physiological response of a human. Much further study of the metabolome of medicinal plants has enormous potential for the discovery of new, neurologically active drugs for treatment and prevention of human diseases, but may also lead to new understandings of the control of plant growth and development.

10.3 Neurotoxins in Plants

There is another group of neuroregulating compounds that includes neurotoxins and allergens and the investigation of these compounds can provide new insights into some of the most chronic and devastating human diseases. Many of these toxic plant compounds are unusual non-protein amino acids



ß-oxalylaminoalanine (BOAA)



ß-methylaminoalanine (BMAA)

Fig. 10.6. Neurotoxic, non-protein amino acids from plants

that can inhibit protein synthesis, disrupt urea cycle function or impair neurotransmission (Bell 2003). One example of a neurologically damaging amino acid from plants is β-oxalylaminoalanine (BOAA) found in *Lathyrus sativus*. *L. sativus* (grasspea or chickling vetch, guaya in Ethiopia, khesari in India) is a small legume grown and eaten throughout many parts of the world. Consumption of seeds high in BOAA is thought to be the cause of lathyrism, a neurodegenerative disease characterized by spastic paraparesis. Taken in small quantities, pigeonpea is not neurotoxic but during droughts or when other food is limited, epidemics in lathyrism have occurred (Spencer, 1999). In 1977 an outbreak of lathyrism in Ethiopia caused more than 2,500 people to become ill (Spencer and Palmer 2003). Both BOAA and a related compound BMAA (Fig. 10.6) can damage neurons but the potency and specificity of these amino acids is quite low (Lindstrom et al. 1990). The compounds are classified as excitotoxins, a toxic molecule that overstimulates stimulates neurons resulting in cellular damage or cell death. In cell cultures, both BOAA and β-methylaminoalanine (BMAA) injure neurons in a dose-dependent manner and require various metabolic cofactors such as bicarbonate (Weiss et al. 1989). As a result of both the general complexity of progressive neurodegenerative diseases and the mechanisms of action of compounds that are not acutely toxic, the study of plant-based neurotoxins requires an interdisciplinary team of neurologists, chemists and botanists. One example of this type of research is the study of the epidemic of progressive neurodegenerative disease on Guam.

On Guam and in other Pacific locales, indigenous residents and immigrants have experienced an epidemic of progressive neurodegenerative diseases that manifests as atypical parkinsonism, dementia, motor neuron disease, or a combination of these three phenotypes. The Chamorros of Guam call the disease lytico-bodig, while neuroscientists refer to it as the amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC). In the 1950s, at the height of the epidemic, ALS/PDC was 100 times more prevalent than ALS elsewhere in the world (Kurland and Mulder 1954; Mulder et al. 1954). The disease of Chamorros is clinically and pathologically like classical ALS, with additional similarities to Alzheimer's disease, and Parkinson's disease. It has often been called the "Rosetta Stone" of neurological disease as it is thought that understanding ALS/PDC on Guam may provide the keys to understanding progressive neurodegenerative diseases elsewhere. The exact cause of the disease remains uncertain but epidemiological research on Guam has implicated environmental factors rather than genetic predisposition, infectious agents or mineral imbalances. At the climax of the epidemic ALS/PDC was the main cause of death of adult Chamorros but the incidence of the disease has steadily declined in recent decades, potentially as a result of western influences on Chamorro culture. Today the disease occurs only in older people, and rarely in any individual born after 1960. The study of this older generation of Chamorros and the Chamorro culture is essential to understanding of the disease and the search for disease causes.

In 1967, Kurland and Whiting began ethnobotanical studies to determine if the Chamorro culture, lifestyle and diet might be implicated as the cause of ALS/PDC. Flour made from the seeds of the indigenous cycad (*Cycas micronesica* Hill.) was extensively investigated. The Chamorros knew the cycad seeds to be acutely toxic and detoxified the flour made from seeds by multiple washings over a 3-week period. Chemical examination of the cycad flour and cycad seeds led to the discovery of the unusual non-protein amino acid BMAA (Vega and Bell 1969). BMAA is distributed across all tissues in cycad plants but is most concentrated in the reproductive tissues where it potentially protects gametes from herbivory and has ensured the survival of the species over three hundred million years (Banack and Cox 2003a). Interestingly, new information indicates that the neurotoxin BMAA may also play a role in plant physiology. A recent study with *Arabidopsis* seedlings demonstrated a 2–3-fold increase in hypocotyls elongation and inhibition of cotyledon opening when seeds were germinated in the presence of low concentrations of BMAA (Brenner et al. 2000).

In animal systems, BMAA has an established toxicity of about 0.4 mg/g (Polsky et al. 1972). Spencer et al. (1986) suggested that BMAA might be a cause of ALS/PDC and fed monkeys with large doses of BMAA, causing acute damage to motor neurons in the spinal cord producing a flaccid

paralysis, and damaged neurons in the striatum and cortex producing parkinsonian and behavioral changes. However, Duncan et al. (1988) argued that humans would need to eat massive amounts of cycad seed flour on a daily basis to generate a similar progressive neurological disease and as a result the BMAA hypothesis was largely discounted. In 2002, Cox and Sachs hypothesized that biomagnification of BMAA through the Guam ecosystem could effectively concentrate cycad neurotoxins and increase the exposure of the Chamorro people. The hypothesis suggested that flying foxes (*Pteropus mariannus mariannus*), large indigenous bats that feed on cycad seeds, biomagnified cycad neurotoxins in the Chamorro diet. In interviews many Chamorros identified flying foxes as the most prized food item. The population of flying foxes dramatically declined from about 70,000 in the 1920s to just 58 individuals currently alive in Guam (Monson et al. 2003). Flying foxes consume the outermost integument of cycad seeds, where BMAA is present at the highest concentrations, masticate the tissue to extract the juices and spit out the pulp as "ejectapellets," thereby effectively concentrating the neurotoxin (Banack and Cox 2003b).Varying concentrations of BMAA in individual flying foxes, related to individual foraging patterns, might have resulted in different cumulative doses of neurotoxins among Chamorros who consumed equivalent numbers of flying foxes (Banack and Cox 2003b). Ecological surveys of cycads on Guam revealed that cycads have modified root structures that are positively geotropic and house a cyanobacterial symbiont (Cox et al. 2003). Axenic cultures of nostoc isolated from "coralloid" roots of cycads produced about 0.3 μg/g of BMAA (Cox et al. 2003) and BMAA was found in the roots with heavy cyanobacterial infections at concentrations up to 37 μg/g (Murch et al. 2004c). Recently, BMAA was also found in diverse taxa of cyanobacteria collected worldwide (Cox et al. 2005). Further, BMAA was detected in the brain tissues of Chamorro patients who died of ALS/PDC and in the brain tissues of two Canadians who died of Alzheimer's disease (Murch et al. 2004d) and chemical analysis of the Chamorro flour and flying fox samples from Guam revealed that although BMAA is a non-protein amino acid, it is accumulated into proteins in cyanobacteria, cycads, flying foxes and humans (Murch et al. 2004c). These data are fascinating as together they describe the biomagnification and persistence of a naturally occurring neurotoxin through various trophic levels of the ecosystem culminating with human exposure.

10.4 Conclusions and Future Prospects

The study of plant cell communications and signaling mechanisms is inherently problematic. Signaling molecules are highly reactive, present in small concentrations and have short lifetimes within tissues. Similarly, receptors of signals within plants can be unstable, short-lived and highly reactive. A variety of specific signals, amplification mechanisms, ion channels and gene transcriptions are likely involved in any morphological change. Frequently we are left to interpret the morphological outcome of a cascade of metabolic reactions as the result of a single induction and the various regulatory events at different steps in the metabolic cascade remain undefined. This is the case with many of the auxin- or cytokinin-induced morphological responses. The advent of new technologies makes possible the generation of metabolic snapshots at specific points in time and will help in the generation of a clearer picture of the metabolic events in plant growth, development and morphogenesis. However, there is a need for better model systems and new approaches to plant development research that are more inclusive of intercellular signaling in plants. Together, the development of new technologies and new model systems will allow for rapid advancements in the next decade that will further our understanding of the role of neurologically active compounds in plants. It is likely that mechanisms of circadian rhythms are highly conserved across species and will be found to profoundly influence plant life. Similarly, many of the compounds currently considered to be secondary metabolites may actually be essential for the growth and development of plants and for plant survival. Finally, metabolome analysis of higher plants is revealing more chemical diversity than was anticipated and will continue to provide novel compounds for prevention and treatment of neurological disorders.

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11 Amino Acid Transport in Plants and Transport of Neurotransmitters in Animals: a Common Mechanism?

Tobias Müller, Wolfgang Koch, Daniel Wipf

Abstract Amino acids (AA) are essential elements in animals and plants. In a genome-wide analysis in yeasts, plants and animals five AA transporter superfamilies could be identified. Transporters of one superfamily (ATF1, SLC38) which includes animal and plant members correspond to proteins involved in neurotransmitter transport in animals. Their close relation with plant genes suggests that a closely related transport mechanism could be involved in AA transport in plants. This chapter summarizes current knowledge on AA transport in animals and plants and the question of a possible common mechanism is discussed.

11.1 Introduction

Amino acids (AA) are essential elements in animals and plants. They are involved in several functions such as protein synthesis, hormone metabolism, nerve transmission, cell growth, production of metabolic energy, nucleobase synthesis, nitrogen metabolism and urea biosynthesis. AA transporters were characterized physiologically in animals as well as in plants. In a genome-wide analysis in yeasts, plants and animals Wipf et al. (2002) identified five AA transporter superfamilies. Two superfamilies (preferentially Na^+-coupled transport) were only found in animals, whereas the other three superfamilies have members in both animal and plant genomes (Wipf et al. 2002). It is worthwhile noting that transporters of one superfamily which includes animal (SLC32, 36 and 38, Sects. 11.2.5–11.2.7) and plant (AA transporter family 1, ATF1, Sect. 11.3.2) members correspond to proteins involved in neurotransmission in animals. The mechanisms of neurotransmission are well described and the close relation with plant genes suggests that a closely related transport mechanism could be involved in AA transport in plants. This chapter summarizes current knowledge on neurotransmitter transport in animals and AA transport in plants and the question of a possible common mechanism is discussed.

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11.2 Amino Acid Transport in Animals

The proteins involved in AA transport are found in at least seven solute carrier families (SLC): SLC7, SLC17, SLC32, SLC36 and SLC38 are phylogenetically related to plant transport systems, whereas no homologs to SLC1 and SLC6 could be found in the *Arabidopsis* genome.

11.2.1 Sodium Dicarboxylate Symporter Family (SDS, SLC1)

The SLC1 family comprises five high-affinity glutamate transporters (system X^{-}_{AG}) SLCA1 (EAAC1, EAAT3), SLC1A2 (GlT1, EAAT2), SLC1A3 (GLAST, EAAT1), SLC1A6 (EAAT4) and SLC1A7 (EAAT5) and two neutral AA transporters (system ASC) SLC1A4 (ASCT1, SATT) and SLC1A5 (ASCT2, AAAT, hATB0) (Kanai and Hediger 2004). Uptake of glutamate is coupled with cotransport of three sodium ions, one proton and the countertransport of one potassium ion (Fig. 11.1) (Zerangue and Kavanaugh 1996). This coupling makes the transport against a concentration gradient more powerful and speeds up the removal of excitatory AA from the synaptic cleft and in the surrounding neuroglia, protecting neurons against accumulation to neurotoxic levels (Fig. 11.1) (Kanai and Hediger 2004). The glutamate transporter EAAC1 was also shown to facilitate substrate exchange (Kanai and Hediger 2004). SLC1 members regularly have ten transmembrane domains with cytosolic C- and N- termini (Wipf et al. 2002). Recently Kanai and Hediger (2004) developed a membrane model of glutamate transporters with eight predicted transmembrane domains, a large extracellular glycosylated loop and a reentrant loop (two shorter hydrophobic domains) between domains 7 and 8, similar to the ion-permeating pore of ion channels. The neutral AA transporter isoforms (ASCT1 and ASCT2; Fig. 11.1) transport L-alanine, L-serine, L-cysteine and L-threonine with high affinity in dependency to sodium ions. In addition to these substrates, ASCT2 also has a high affinity to glutamine and asparagine and a lower affinity to methionine, leucine and glycine (Kanai and Hediger 2004). One of the main functions of ASC transporters is the exchange of AA, but they can also act as ligand-gated ion channels without being combined with potassium countertransport and proton cotransport (Broer et al. 2000; Zerangue and Kavanaugh 1996). Most SLC1 members are found in brain tissues although representatives of system B^0 (Lynch and McGivan 1987), ASC (Vadgama and Christensen 1984), ASCT1 and ASCT2 (Zerangue and Kavanaugh 1996) and X^{-}_{AG} have also been found in nonbrain tissues (Wipf et al. 2002). For

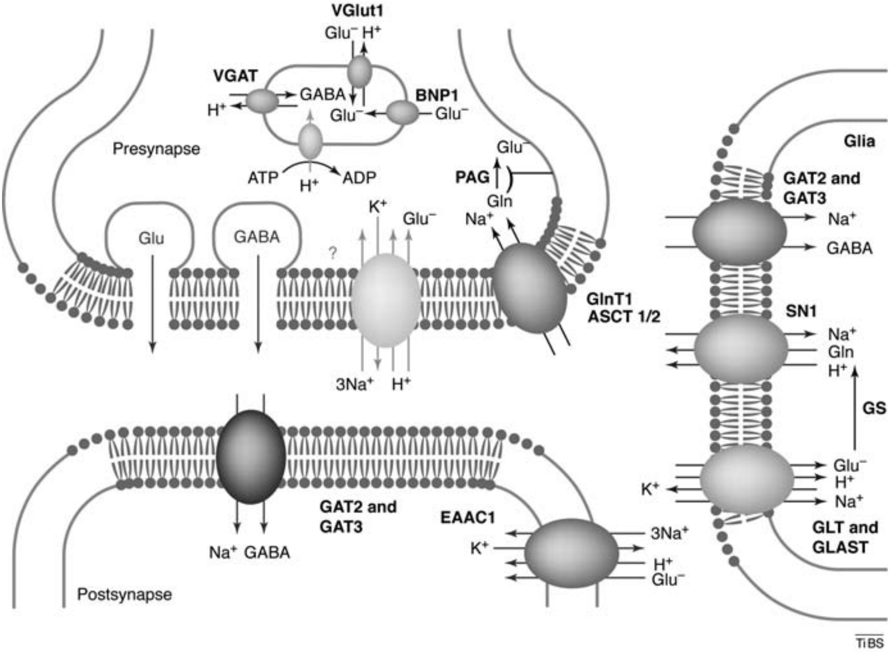

Fig. 11.1. Glutamate and γ-aminobutyric acid (*GABA*) transporters at synapses. The excitatory neurotransmitter glutamate and the inhibitory neurotransmitter GABA are stored in synaptic vesicles at presynaptic terminals and released into the synaptic cleft to act on postsynatptic receptors. High-affinity transporters play an important role in removing glutamate and GABA released from the synaptic cleft and maintaining the external glutamate and GABA concentrations below neurotoxic levels (*EAAC1*, sodium dicarboxylate symporter superfamily) for glutamate, *GAT2* and *GAT3* (neurotransmitter superfamily) for GABA). Glutamate taken up in astroglial cells by *GLT* and *GLAST* (sodium dicarboxylate symporter superfamily) is degraded to glutamine by the glutamine synthetase (*GS*). Glutamine, which can serve as a precursor of glutamate synthesis in the nerve terminal, is exported from the glial by *SN1* (amino acid, *AA*, transporter superfamily 1, *ATF1*). In the neuron glutamine [taken up by *GlnT1*, *ASC1* and *ASC2* (sodium dicarboxylate symporter superfamily)] is converted to glutamate by a phosphate-activated glutaminase (*PAG*). Neurotransmitters are transported into synaptic vesicles by *BNP1* and *VGluT1* (major facilitator superfamily) for glutamate, and *VGAT* (ATF1) for GABA. Until now it is not clear whether the presynaptic terminal possess a specialized glutamate transporter. (Reprinted from Wipf et al. 2002, with permission from Elsevier)

example, EAAT5 (system X^{-}_{AG}) is reported to be expressed primarily in the retina (Arriza et al. 1997), suggesting that the EAAT5 protein is involved in visual processes (Kanai and Hediger 2004). No SLC1 homologs were reported in plants.

11.2.2 The Sodium- and Chloride-Dependent Neurotransmitter Transporter Family (NTF, SLC6)

Members of the neurotransmitter superfamily (SLC6) are key regulators of extracellular neurotransmitter levels and are necessary for normal neurotransmission (Fig. 11.1) (Melikian 2004). SLC6 transporters (e.g., γ-aminobutyric acid, GABA, transporters GAT1-3, Fig. 11.1, dopamine transporter, DAT, etc.) regulate the level of extracellular solute concentrations and are mainly found in the central and peripheral nervous system (Chen et al. 2004) where they are involved in the signalling pathway. They are, however, also found in many nonneural tissues with diverse physiological functions. For example, SLC6A6 (taurine transporter, TAUT) and SLC6A12 (betaine transporter, BGT1) take part in osmoregulation in kidney (Chen et al. 2004). Genes of the SLC6 family encode proteins of approximately 600 AA with a common structure of 12 transmembrane domains with predicted intracellular N- and C-termini (Nelson 1998). The solute transport is coupled with the cotransport of Na^+ and Cl^-. The transport of neurotransmitter molecules against the concentration gradient uses energy produced by the electrochemical Na^+ gradient (Chen et al. 2004; Wipf et al. 2002). Na^+ is absolutely necessary for transport activities in contrast to Cl^-, whose requirement for cotransport varies across members (Chen et al. 2004). Most of the SLC6 members are regulated by protein kinases, although some other mechanisms are assumed to exist (Blakely and Bauman 2000). In organisms like yeasts, fungi and plants, that do not typically use Na^+ gradients for metabolite uptake, no homologs to the SLC6 family could be found (Saier 1999).

11.2.3 Cationic Amino Acid Transporters and Heteromeric Amino Acid Transporters (SLC7)

The SLC7 family is divided into two subgroups: cationic AA transporters (CAT) (SLC7A1–4) and heteromeric AA transporters (HAT) (SLC7A5–11). Members of the CAT subgroup have 14 putative transmembrane domains and are found in both animals and plants (Wipf et al. 2002). Mammalian CATs mediate Na^+-independent uptake of cationic AA (Closs et al. 1993). Transport properties of the CAT are those of system y^+. The second subgroup (HAT) comprises proteins with 12 putative transmembrane domains. In contrast to what was observed for the CAT members, HAT transporters are quite diverse in terms of substrate selectivity, transporting neutral L-AA (large and small), negatively charged AA and cationic AA plus neutral

AA (Verrey et al. 2004). Mammalian HATs are composed of a light subunit (LAT-1, y^{+}LAT1, y^{+}LAT-2, LAT-2, $b^{o,+}$AT, Asc-1, xCT and two orphans) and a heavy subunit ("heavy chain, SLC3") (Ganapathy et al. 2001, Palacin et al. 1998). The heavy subunit is required for the generation of active plasma membrane AA transporters. This subunit is glycosylated, contains only one putative transmembrane domain and plays a role in trafficking to target membranes, for example, in polarized epithelial cells (Palacin et al. 1998). Six of the known light chains associate with 4F2hc, the widespread heavy subunit, strictly basolateraly localized in epithelia (Verrey et al. 2004). A second heavy subunit, the brush border localized rBAT, was identified in mammals. So far, SLC3 homologs have not been found in plants.

11.2.4 The Type I Phosphate Transporter Family (SLC17)

The transport of organic anions is the primary function of the SLC17 family, which contains four type I phosphate transporters (SLC36A1–4), a lysosomal sialic acid transporter (SIALIN, SLC36A5) and, surprisingly, three vesicular glutamate transporters [VGLUT1 (or BNP1), VGLUT2–3, (SLC36A6–8)] occurring in synaptic vesicles (Fig. 11.1) (Reimer and Edwards 2004). All three VGLUT have been shown to have a K_{m} for glutamate around 1 mM (Bai et al. 2001; Bellocchio et al. 2000; Fremeau et al. 2002). Homologs of SLC17 members have been identified in plant genomes without being more closely characterized (Reimer and Edwards 2004) (Sect. 11.3.2).

11.2.5 The Vesicular Inhibitory Amino Acid Transporter Family (VIAAT, SLC32)

This family includes a single member, the vesicular inhibitory AA transporter (VIAAT) or vesicular GABA transporter (VGAT, Fig. 11.1) (Gasnier 2004). VGAT is localized on synaptic vesicles (Fig. 11.1), where it mediates the uptake of GABA and glycine into synaptic vesicles and their exocytotic release (Gasnier 2004; McIntire et al. 1997).

11.2.6 The Proton/Amino Acid Transporter Family (PAT, SLC36)

The SLC36 family comprises four members which encode around 500 AA proteins and have 11 predicted transmembrane domains (Boll et al. 2004).

The first one (SLC36A1, LYAAT1, PAT1) was identified as a lysosomal AA transporter, which mediates proton-coupled export of AA from lysosomes (Sagne et al. 2001). LYAAT1 was also localized to the apical membrane in intestine cells, where it mediates proton-driven uptake of small neutral AA (Thwaites et al. 1995). Like PAT1, SLC36A2 (PAT2) mediates proton symport uptake of small neutral AA. In addition, the SLC36 family comprises two orphans, SLC36A3 and SLC36A4.

11.2.7
The Sodium-Coupled Neutral Amino Acid Transporter Family (SNAT, SLC38)

In mammals transporters from the SLC38 family play various roles. For example, a role for SNAT2 in the provision of AA entering the gluconeogenic pathway could be suggested. SNAT3 may play a role in the response to acidosis in rats (Karinch et al. 2002) or could be involved in the detoxification of ammonia from the portal blood in the liver (Mackenzie and Erickson 2004). Recently Mackenzie and Erickson (2004) renamed the SLC38 members SNAT1–6. SNAT stands for sodium-coupled neutral AA transporter and also recalls the classic transport activities, i.e., system N/A transporter. SNAT1 (former ATA1, GlnT, NAT2, SA2, SAT1), SNAT2 (former ATA2, KIAA1382, SA1, SAT2) and SNAT4 (former ATA3, FLJ10191, NAT3, PAAT, SAT3) belong to the system A subfamily transporting aliphatic AA in correlation with an uptake of Na^+ (Mackenzie and Erickson 2004).

System A members are pH-sensitive and underlie a sophisticated hormonal and adaptive regulation (Häussinger and Kilberg 1992; McGivan and Pastor Anglada 1994; Shotwell et al. 1981). SNAT3 [former g17, NAT (mouse), SN1; Fig. 11.1 and SNAT5 (former JM24, SN2) correspond to a second kind of system, namely, system N. SNAT3 and SNAT5 are like system A Na^+-dependent. Glutamine, histidine and asparagine belong to the substrates transported by system N, whereas system A is not that limited, transporting alanine, asparagine, cysteine, glutamine, glycine, methionine and serin (Mackenzie et al. 2004). Transport is also coupled with a H^+ countertransport which produces a low extracellular pH that inhibits the system (Albers et al. 2001; Broer et al. 2002; Chaudhry et al. 1999, 2001, 2002; Nakanishi et al. 2001). As in system A, the regulation of system N is adaptive (Jacob et al. 1986; Kilberg et al. 1980). SNAT6 [former NAT-1 (human)], the orphan member of the SLC38 family has been isolated from a human brain complementary DNA (cDNA) library (Mackenzie and Erickson 2004).

Note that SLC32, SLC36 and SLC38 members can be grouped in an eukaryotic specific superfamily, namely the ATF1 superfamily (Sect. 11.3.2) (Wipf et al. 2002; Fig. 11.3).

It is important to mention that further AA transporters exist in animals. TAT1, a SLC16 (monocarboxylate transporter family) member specifically is an aromatic AA transporter (Kim et al. 2001). Members of the mitochondrial carrier family (SLC 25) potentially involved in AA transport are present in animals and plants (Wipf et al. 2002).

11.3 Amino Acid Transport in Plants

For the distribution of AA in the plant several transport steps across membranes are necessary. AA have to cross the plasma membrane when taken up from the soil into root cells. In mycorrhized plants AA that pass the fungal layer or are produced within the fungus must be taken up from the plant root cells. AA synthesized in root tissue have to be exported to the shoot via xylem. Since mature xylem elements are dead cells, AA need to cross the plasma membrane to enter the xylem, potentially via facilitators, exchangers or antiporters. From the site of biosynthesis within the plant, mainly in the plastids of the leaf or root, AA have to pass several membranes to enter the long-distance traffic routes. Plastids are surrounded by two membranes, an outer and an inner envelope. AA cross the outer envelope of plastids via outer envelope proteins 16 and 14, which form channels permeable to amines and AA (Pohlmeyer et al. 1997, 1998). For the inner envelope, no AA transporters have been described so far. To leave the cytoplasm of the cell, AA have to pass the plasma membrane. The concentration of the AA in mesophyll cytoplasm and in the phloem is significantly higher compared with that in the apoplasm, suggesting an active transport of AA into the phloem (Lohaus et al. 1995). In feeding experiments it was shown that large parts of AA fed directly to the xylem sap appear unchanged in the phloem sap. This indicates that AA can be exchanged between xylem and phloem (Atkins 2000; Pate and Sharkey 1975) and can cycle within the plant passing the membranes of multiple cells via transporters.

11.3.1 Amino Acid–Polyamine–Choline Transporter Family

Uptake of AA is well characterized in yeasts (Fischer et al. 1998), where 24 transporters of the AA–polyamine–choline (APC) family (pfam00324) have been functionally characterized. The members of the APC family cluster in subgroups reflecting the three kingdoms (yeast, plant and animal) (Fig. 11.2) (Wipf et al. 2002). Mammalian CATs are representatives of system y^+, mediating Na^+-independent uptake of cationic AA (Closs

et al. 1993), and are characterized by a high affinity for cationic AA. The plant membranes maintain a proton gradient over the plasma membrane via plasma membrane ATPases, and, correspondingly, the first plant member of the APC family, *At*CAT1, mediated the accumulation of histidine in a pH-dependent manner (Frommer et al. 1995). However, it remains unclear whether *At*CAT1 functions as a uniporter or an exchanger, or as a secondary active proton cotransporter. Recent studies of a related protein, *At*CAT5, showed that the transport of basic AA mediated by *At*CAT5 is sensitive to protonophores, indicating that at least *At*CAT5 is a proton symporter. *At*CAT5 is a high-affinity transporter for arginin with an apparent K_m value of 12 μM (Su et al. 2004). Other basic AA were good competitors for ^{14}C arginine uptake into yeast cells expressing *At*CAT5, while neutral or acidic AA like aspartate, proline, glutamate and glutamine were less effective in uptake inhibition. The transport selectivity of *At*CAT5 is similar to that of *At*CAT1, which also transports basic AA preferentially.

For other members of the CAT-family in *Arabidopsis* no direct transport activity was demonstrated, nevertheless *At*CAT3 and *At*CAT6 expressed in yeast cells increased toxicity to the toxic analogue of glutamine, (*S*)-2 amino-6-diazo-5-oxo-L-norleucine, but not to the arginine analog canavanine (Su et al. 2004). The fact that arginine is not a substrate for *At*CAT3 and *At*CAT6 already shows that not all members of the CAT family in *Arabidopsis* can be supposed to be cationic AA transporters.

In animals, the APC-type AA transporters require an additional subunit for functional expression at the plasma membrane (Sect. 11.2.3). Members of the mammalian CAT family essentially transport cationic AA by facilitated diffusion, while the HATs are in contrast quite diverse in terms of substrate selectivity and function as obligatory exchangers. In plants no homologs of secondary subunits have yet been identified, and one subbranch of the APC family, the LAT (Fig. 11.2), have still not been functionally characterized in plants.

11.3.2 Amino Acid Transporter Family 1

Members of the ATF1 family were first described in plants as functional AA transporters. Structurally related proteins were identified in yeasts and animals (Fischer et al. 1998). The superfamily contains plant-specific subbranches, and branches that are more structurally related to mammalian transporters (Fig. 11.3). Within the plant-specific subbranches, the best-characterized members are the *Arabidopsis* AA permeases (AAP; Figs. 11.3, 11.4). Several AAP have been isolated from *Arabidopsis* by complementation of yeast transport mutants with plant cDNAs (Fischer et al.

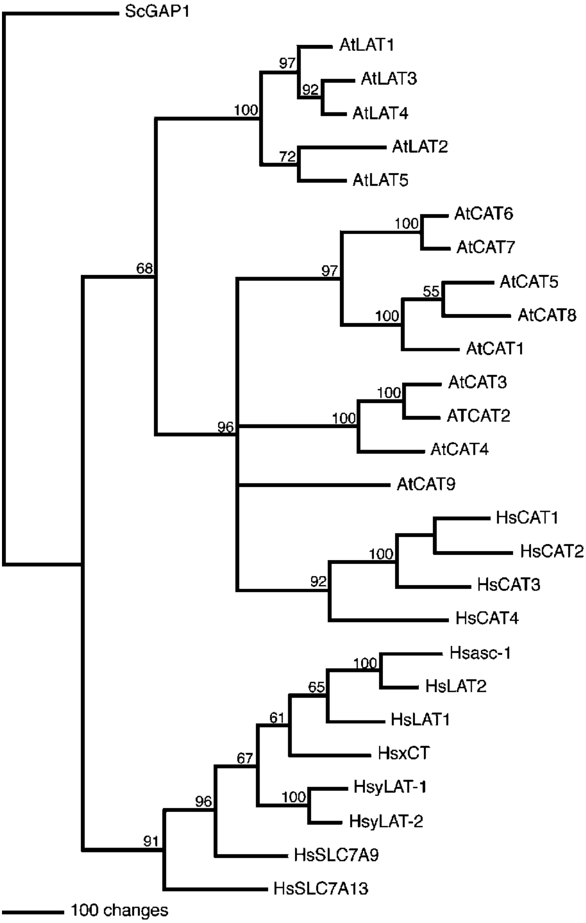

Fig. 11.2. Phylogenetic tree of the AA–polyamine–choline (*APC*) superfamily (SLC7) (*Hs Homo sapiens*, *At Arabidopsis thaliana*). Maximum parsimony analyses were performed using PAUP 4.0b10 (Swofford 1998). Heuristic tree searches were executed using 100 random sequence additions and the tree bisection–reconnection branch-swapping algorithm with random sequence analysis. The complete alignment was based on 691 sites; 586 were phylogenetically informative. Bootstrap values (%) are indicated at branch nodes

1995; Frommer et al. 1993; Hsu et al. 1993; Kwart et al. 1993; Rentsch et al. 1996). AAP mediate H^+-coupled uptake of a wide spectrum of AA when expressed in oocytes (Fischer et al. 2002). *At*AAP1-5 represent low-affinity transporters with low selectivity towards AA side chains; only AAP3 and AAP5 transport basic AA efficiently. AAP6 has an approximately tenfold higher affinity towards all substrates when compared with other AAP. For glutamine, glutamate and asparagine, major transport forms of organic

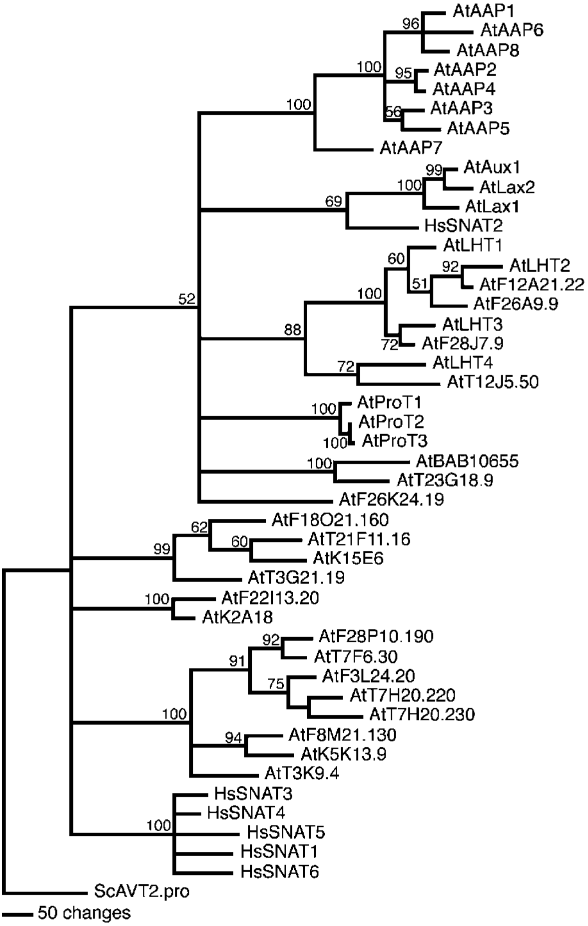

Fig. 11.3. Phylogenetic tree of the ATF1 superfamily. Maximum parsimony analyses were performed using PAUP 4.0b10 (Swofford 1998). Heuristic tree searches were executed using 100 random sequence additions and the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. The complete alignment was based on 529 sites; 498 were phylogenetically informative. Bootstrap values (%) are indicated at branch nodes

nitrogen, these transporters have K_m values that are in a physiological range with respect to the concentrations of these AA found in the phloem and xylem. Aspartate is transported by most AAP with an extremely low affinity and efficiency. Only AAP6 has a K_m value for aspartate that is in a physiological range (Fischer et al. 2002).

Plants take up nitrate and ammonium but also AA as a nitrogen source and, more importantly, AA are the principal long-distance transport forms of organic nitrogen. In accordance with these facts, reporter gene analyses

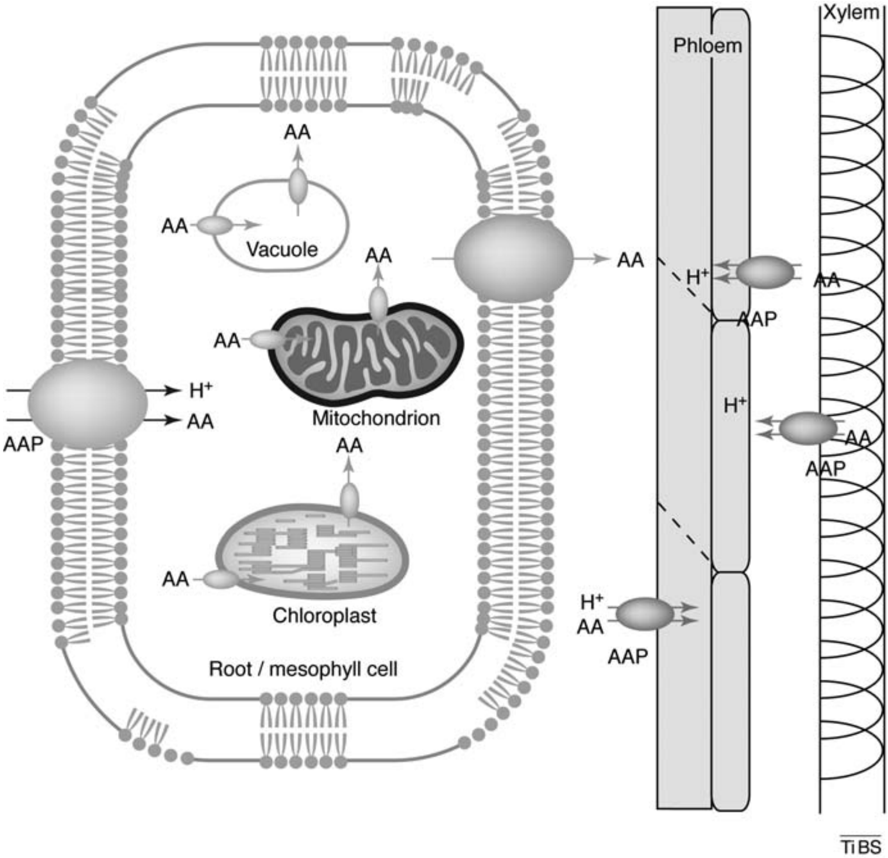

Fig. 11.4. AA transporters in plants. Although a wide spectrum of proteogenic AA accumulate in phloem (100–200 mM) and xylem sap (about tenfold lower concentration than in phloem), amines and AA predominate. The actual intracellular, apoplasmic, and phloem composition of AA is thought to be similar, thus the relatively unspecific AA permeases (*AAP*) might be responsible for functions like uptake of AA from the xylem into the cell and phloem loading. No organellar transporter has been identified to date. (Reprinted Wipf et al. 2002, with permission from Elsevier)

showed that the members of the AAP family are associated with the vascular tissue or the reproductive organs (Hirner et al. 1998; Okumoto et al. 2002, 2004). The properties of AAP described thus match the activities expected for transport proteins involved in moving a wide spectrum of AA into the phloem sap and are consistent with the properties described for uptake kinetics measured in plasma membrane vesicles of leaves (Li and Bush 1990a, b). Antisense repression of a related AA transporter from potato, *St*AAP1, expressed in source leaves led to reduced concentration of

AA in potato tubers. This reduced transport to sink organs supports the hypothesis of a role in phloem loading for some of the AAP (Koch et al. 2003).

Besides long-distance transport the delivery of reduced nitrogen to the reproductive organs is a key factor for seed development. The import of AA is an essential prerequisite for seed development since the accumulation of storage proteins must be preceded by AA import. It could be shown that the expression of the AA transporter in seeds indeed precedes the expression of the storage protein *At*S1 in *Arabidopsis* (Hirner et al. 1998). A similar increase in the expression of a legume AA transporter has been shown in *Vicia faba* (Miranda et al. 2001). In pea, it has been demonstrated that *Ps*AAP1 is expressed in the transfer cell layer of cotyledons and might be responsible for taking up AA released from the seed coat (Tegeder et al. 2000). Ectopic overexpression of an AAP in pea seeds increases AA uptake and increases storage protein content by 40–50%. The results suggest that nitrogen supply limits protein synthesis and storage in the seed (Rolletschek et al. 2005).

From the second plant-specific branch of the ATF family, the LHT (Fig. 11.3), two members have been functionally characterized so far. *At*LHT1 has been described as a transporter specific for lysine and histidine, but other AA are also recognized (Chen and Bush 1997). LHT1 is present in all tissues, with expression being strongest in young leaves, flowers and siliques. Recent characterization of a related protein, LHT2, shows that this transporter is more likely to be a general AAP with a high affinity for proline ($K_m \sim 10\,\mu M$) and aspartate ($K_m \sim 72\,\mu M$). Inhibition studies indicate potentially similar affinities towards most AA except the basic AA histidine, lysine and arginine (Lee and Tegeder 2004). Expression of the gene in the tapetum tissue of the flower suggests a role in the transfer of organic nitrogen to the tapetum tissue and in supplying the pollen with nutrients. The affinity of LHT2 towards proline is a factor of 10 higher than the affinity of AAP6 and is, together with the high affinity of CAT5 for arginine, among the highest determined for AA transporters in plants. AA concentrations in phloem, xylem, and supposedly in mesophyll cells, are highly variable depending on carbon supply, inorganic nitrogen and light. Thus, it makes sense that the relatively immobile plants can respond to variations in AA concentrations with transporters that have different K_m values and transportation speed.

ANT1, the only member characterized of a more distantly related branch (Fig. 11.3) to the AAP and LHT mediates the transport of aromatic and neutral AA (Chen et al. 2001). The substrate specificity found in the third plant-specific branch of the ATF-family, the proline transporters (ProT; Fig. 11.3) is different. In contrast to the plant carriers just described, ProT preferentially transport the compatible solutes proline (K_m values of 0.427, 0.500 and 1 mM for *At*ProT1–3, respectively), betaine (K_m values of 0.115, 0.267 and 0.290 mM for *At*ProT1–3) and GABA (4.5, 4.01 and 5.12 mM)

(Grallath et al. 2005; Rentsch et al. 1996). *At*ProT1 is expressed in the phloem and phloem parenchyma cells, whereas *At*ProT2 expression is restricted to the epidermis and cortex cells of roots. *At*ProT3 finally is only expressed in the aerial parts of the plant, in the epidermal cells of the leaves. Induction of the expression of the *At*ProT upon salt stress implies a role in stress adaptation or a function in organs of plants that desiccate like pollen and seeds (Rentsch and Frommer 1996; Schwacke et al. 1999). In mangroves, where salt concentrations are permanently high, the transport of compatible solutes is thought to be an important factor for adaptation to the environment (Waditee et al. 2002).

In plants nothing is known about the compartimentation of AA or the transport steps into the vacuole. Some plant ATF1 members might fulfil a function in import or export of solutes in vesicles and vacuoles. A similar mechanism like the concentration of GABA in synaptic vesicles via VGAT (Fig. 11.1) is possible for the related uncharacterized plant proteins in the ATF family.

The plant members of the ATF family investigated so far all function as H^+-coupled systems, probably playing a role in accumulating AA within the plant cell. Subcellular localization studies could help to identify the transporters involved in the compartimentation of AA into the vacuole.

11.4 Conclusions and Future Prospects

Taken together, plant AA transporters can be grouped in three superfamilies with homologs from the animal kingdom (Lalonde et al. 2004; Wipf et al. 2002): (1) the ATF1 superfamily (SLC32, SLC36 and SLC38 and *Arabidopsis* ATF1 members) (Fig. 11.3); (2) the APC superfamily (SLC7, *At*CATs and *At*LATs) (Fig. 11.2); (3) AA transporters within the major facilitator superfamily (MFS) (SLC17, homologs of unknown function in *Arabidopsis* (Wipf et al. 2002).

As described, several export steps are required for AA distribution in plants (Fig. 11.3): phloem-to-xylem transfer, release of AA into the apoplast of the leaf mesophyll as the first step for phloem loading, unloading in sink organs, supply of symplasmic isolated cells, e.g., growing embryo, guard cells and pollen. Multiple possibilities exist, including vesicular transport or carrier-mediated transport, but until now no exporter has been identified in plants. Vesicular export is well established for AA and analogs in mammalian nerve cells. Carriers for the active uptake of AA into secretory vesicles have been identified (Fig. 11.1) and homologous plant genes exist (Fig. 11.3). Furthermore, certain mammalian AA transporters are implicated in cellular export at the plasma membrane, especially from intestinal

cells, and it has been speculated that the corresponding plant homologs might fulfil similar functions (Wipf et al. 2002). Quite recently a mechanism similar to the vesicular secretion of neurotransmitters has been shown for yeasts. A membrane protein localized to internal membranes is responsible for secretion of AA to the surrounding medium in yeasts (Velasco et al. 2004). The recent analysis of an *Arabidopsis* mutant hypersecreting glutamine from hydathodes led to the identification of a new class of membrane proteins possibly involved in this process (Pilot et al. 2004). These proteins, which contain only a single transmembrane domain, might help to identify the mechanism for AA export from plant cells. However, the high number of proteins putatively involved in AA transport in plants (up to 53; Wipf et al. 2002) as well as in animals, and the different mechanisms prevented significant progress. To better understand the mechanisms underlying the loading and unloading of AA from phloem and xylem and the exchange between the two systems, it will be essential to analyse each member individually.

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12 GABA and GHB Neurotransmitters in Plants and Animals

Aaron Fait, Ayelet Yellin, Hillel Fromm

Abstract γ-Aminobutyric acid (GABA) is a four-carbon non-protein amino acid conserved from bacteria to plants and vertebrates. In the latter it is mainly known as a neurotransmitter. The enzymes that synthesize and catabolize GABA constitute a metabolic pathway known as the GABA shunt which bypasses two steps of the tricarboxylic acid cycle. Functional genomics tools using *Arabidopsis* as a model system revealed that the GABA shunt is imperative for normal plant development and for response to stress, and suggest roles for GABA as an important metabolite as well as a potential signaling molecule. Moreover, γ-hydroxybutyrate, a by-product of the GABA shunt and a neurotransmitter in animals, was recently discovered in plants. Here we discuss the possible roles of these two neurotransmitters in plants with focus on components that underlie their roles as signaling molecules.

12.1 Introduction

γ-Aminobutyric acid (GABA) was discovered in plants over half a century ago (Steward et al. 1949), and shortly after it was discovered in vertebrates. Interest in GABA shifted to animals when it was revealed that GABA occurs at high levels in the brain, where it plays a major role in neurotransmission. Interest in the GABA shunt in plants emerged later, following observations that GABA is largely and rapidly produced in response to biotic and abiotic stresses (reviewed by Shelp et al. 1999; Snedden and Fromm 1999; Kinnersley and Turano 2000). A breakthrough in studying GABA in plants came from molecular studies, particularly following the cloning of the Ca^{2+}/calmodulin-regulated GABA-synthesizing enzyme glutamate decarboxylase (GAD; Baum et al. 1993), and later by employing transgenic plants (Baum et al. 1996) and functional genomics tools in *Arabidopsis* to investigate mutants of the GABA metabolic pathway (Bouché et al. 2003, 2004; Palanivelu et al. 2003; Fait et al. 2005).

GABA is mainly metabolized via a short pathway known as the GABA shunt, which bypasses two steps of the tricarboxylic acid (TCA) cycle (Fig. 12.1), and is composed of three enzymes: the cytosolic-localized GAD, and the mitochondrial-localized GABA *trans*-aminase (GABA-T), and succinic semialdehyde dehydrogenase (SSADH). Owing to the fact that most of the knowledge about GABA as a signaling molecule comes from mammalian studies, we will briefly review the subject of GABA signaling in mammals as a basis for discussing aspects of neurotransmitter signaling

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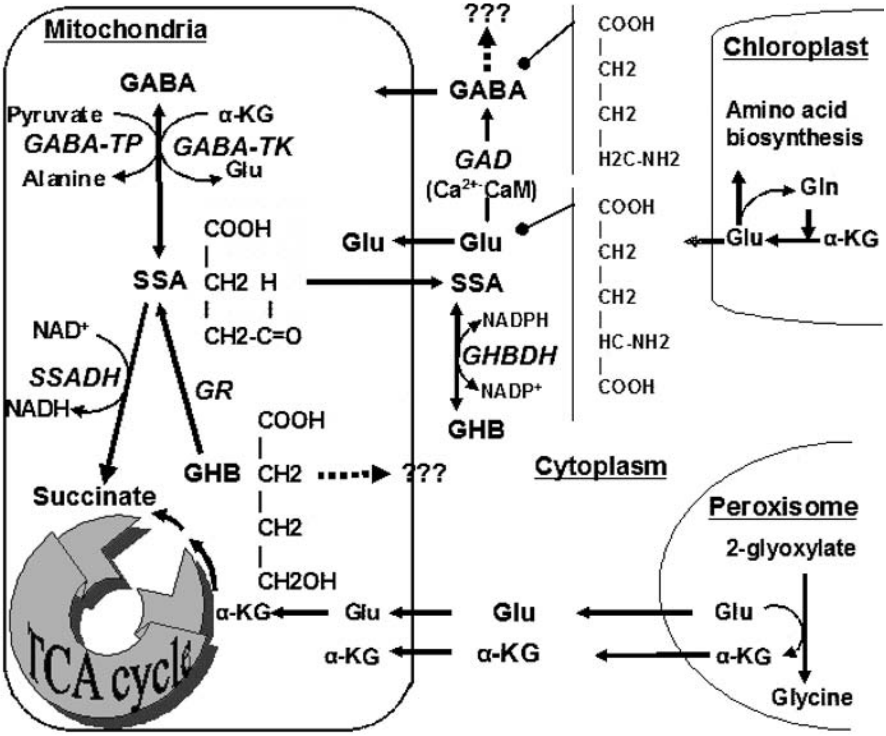

Fig. 12.1. Overview of the γ-aminobutyric acid (*GABA*) shunt and relevant metabolic branches in plants and other organisms. GABA is metabolized via a short pathway known as the GABA shunt, which bypasses two steps of the tricarboxylic acid cycle (*TCA*), and is composed of three enzymes: the cytosolic-localized Ca^{2+}-calmodulin (*CaM*)-regulated glutamate decarboxylase (*GAD*), and the mitochondrial-localized GABA *trans*-aminase (*GABA-T*) and succinic semialdehyde dehydrogenase (*SSADH*). The SSADH substrate succinic semialdehyde (*SSA*) is also catabolized to γ-hydroxybutyrate (*GHB*) by GHB-dehydrogenase (*GHBDH*), which is cytosolic in mammals. GHB catabolism occurs in mammals either in the reverse direction by GHBDH/semialdehyde reductase (see text) or by the mitochondrial-localized L-glucuronate reductase (*GR*; EC 1.1.1.19). In plants, GHB catabolism has not been characterized. Chemical structures are given for GABA-shunt substrates, intermediates, and products. Unknown functions and transport systems are depicted as *dotted arrows* and *question marks*. *Glu* glutamate, *α-KG* α-ketoglutarate, *GABA-TK/P* α-ketoglutaric acid/pyruvate-dependent GABA-T

in plants. We also address the occurrence, in plants, of γ-hydroxybutyrate (GHB), a by-product of the GABA shunt (Fig. 12.1) and a neurotransmitter, which was only recently discovered in plants (Allan et al. 2003; Breitkreuz et al. 2003; Fait et al. 2005). For further details on the different roles of GABA in plants the readers are referred to two recent reviews (Bouché et al. 2003; Bouché and Fromm 2004) and to earlier reviews by Shelp et al. (1999) and Kinnersley and Turano (2000).

12.2 The GABA Shunt and GABA Signaling

12.2.1 Mammalian GABA Signaling

In the central nervous system of mammals GABA is the major inhibitory neurotransmitter. GABA mediates inhibitory synaptic transmission by binding to specialized receptors localized in presynaptic or postsynaptic membranes. Two types of receptors exist in brain cells: ionotropic receptors ($GABA_A$ and $GABA_C$ receptors), which are ligand-gated ion channels, and metabotropic receptors ($GABA_B$ receptors), which are coupled to G-proteins. GABA receptors are also expressed in nonexcitable cells in a variety of human tissues, such as heart, liver, lung, ovary, and testis (Calver et al. 2000). These findings imply that GABA could be a signaling molecule not only in the brain but also in other organs. GABA receptors are also found in lower organisms such as *Caenorhabditis elegans* (Richmond and Jorgensen 1999). Thus, GABA receptors seem to be widely distributed in diverse invertebrate and vertebrate organisms.

In addition to its neurotransmitter function in mature neurons, GABA is involved in the development of the nervous system by promoting neuronal migration, proliferation, and differentiation (reviewed in Owens and Kriegstein 2002). These effects are mediated by the activation of GABA receptors, which provoke depolarization of the membrane in the immature brain, where, in contrast to the adult brain, GABA is excitatory. Indeed, GABA is a chemoattractant that can influence neuronal growth in vitro. During cortical development, GABA can promote DNA synthesis and cell proliferation. Neurons become assembled into functional networks by growing axons and dendrites, collectively called neurites. GABA regulates neuronal differentiation by promoting outgrowth of neurites. In conclusion, in the mammalian brain GABA plays a major role in neural transmission and development, and it functions through interactions with specialized receptors and transporters.

12.2.2 GABA Signaling in Plants

In plants little is known about GABA functions despite the fact it was discovered over half a century ago (Steward et al. 1949). It has been shown that GABA accumulates rapidly in plant tissues exposed to a variety of stresses including acidosis, cold, anoxia, heat, salt, and draught (reviewed by Snedden and Fromm 1999; Kinnersley and Turano 2000). Nevertheless, recent

studies of *Arabidopsis* mutants revealed that disruption of the GABA shunt by functional knockout of the *SSADH* gene causes the accumulation of high levels of reactive oxygen intermediates, necrosis, growth retardation, and hypersensitivity to environmental stresses, including UV radiation and heat (Bouché et al. 2003; Fait et al. 2005). However, from these studies alone it is not clear if GABA functions only as a metabolite en route to the TCA cycle and/or as a signaling molecule by interacting with specialized receptors, and possibly by being transported to specific intracellular compartments or translocated throughout the plant. Further evidence for the possible role of GABA as a signaling molecule in plants came from observations that a gradient of GABA concentration along the pathway of the pollen tube from the stigma to the ovule is imperative for the proper guidance of pollen tubes to the ovule, hence for reproduction (Palanivelu et al. 2003). An *Arabidopsis* mutant of GABA-T (designated *pop2*) showed disruption of this GABA gradient and the concomitant inability of pollen tubes to be directed to the ovule, hence causing infertility. This suggests that GABA plays a role in intercellular signaling in plants, possibly similar to its role in the immature brain of animals. If this is the case, it would imply that GABA interacts with specialized plant receptors, and that GABA-mediated cell–cell communication involves specialized GABA transporters. Moreover, GABA, and known GABA agonists and antagonists affect growth in duckweed (Kinnersley and Lin 2000) and *Stellaria longipes* (Kathiresan et al. 1998). GABA activates arginine decarboxylase in soybean (Turano et al. 1997) and induces gene expression in *S. longipes* (Kathiresan et al. 1998). However, the possible existence of GABA receptors in plants remains elusive. In this regard, genes that are highly homologous to the animal GABA receptors are not found in the *Arabidopsis* genome. However, *Arabidopsis* has a family of 20 genes encoding putative ionotropic glutamate receptors (AtGLRs) (Lacombe et al. 2001) based on their extensive homology with mammal ionotropic glutamate receptors (iGluRs). Interestingly, iGluRs of mammals contain a domain (LIVBP-like domain) which shows structural homology with several receptors, including $GABA_B$ receptors. Therefore, it is tempting to speculate that GABA could bind to this domain and modulate the activity of some of the AtGLRs (Bouché et al. 2003).

In conclusion, there are clues supporting the notion that GABA is a signaling molecule in plants though much remains to be discovered. Further clues about the role of GABA could be obtained by studying the precise dynamic distribution and transport of GABA within plant cells as well as its long-distance translocation in the plant. The issue of neurotransmitter transport in plant and nonplant organisms is discussed in the following.

12.2.3 GABA Transporters

There are two main types of GABA transport systems in living organisms: cell membrane GABA transporters (GATs) and vesicular GABA transporters (vGATs). A list of GABA transporters in different organisms is given in Fig. 12.2. Four complementary DNAs (cDNAs) encoding high-affinity plasma membrane GABA transporters (GATs) have been isolated in rodent and human nervous systems (Borden 1996; Conti et al. 2004). The highly homologous GATs (GAT-1, GAT-2, GAT-3, and BGT-12) exhibit different ionic dependencies and inhibitor sensitivities, and are differentially distributed within the central nervous system (Conti et al. 2004). A vGAT was first isolated from the nematode *C. elegans*, and was termed UNC-47. It is a multipass transmembrane protein with weak sequence homology to plasma membrane amino acid transporters. It is expressed in GABA neurons and is located in the membrane of synaptic vesicles. In addition, the rat homolog of UNC-47 is capable of transporting GABA into vesicular compartments of PC12 cells with the same kinetics of transport that was shown for synaptic vesicles purified from rat brain (McIntire et al. 1997). Moreover, there is a plant homolog of UNC-47 (Fig. 12.2), but no plant homolog was found of any of the GATs. Our bioinformatics search for a GABA transporter in the *Arabidopsis* genome revealed one putative transporter, which resembles the yeast GABA-specific transporter UGA4 (Fig. 12.2).

When *Saccharomyces cerevisiae* GABA transport was characterized, three different GABA permeases were found: the general amino acid permease (GAP1), the proline permease (PUT4), and a specific GABA permease (UGA4). Triple mutant cells (*gap1*, *put4*, *uga4*) grow very poorly on GABA as the sole nitrogen source: their GABA uptake rate is very low under all growth conditions. Expression of one of the three genes is sufficient to restore growth on GABA (Jauniaux et al. 1987). Importantly, the GABA-specific *UGA4* permease gene is activated only when yeast cells are exposed to GABA (Jauniaux et al. 1987; Vissers et al. 1989).

UGA4 is a 62-kDa protein, with 9–12 putative transmembrane regions, which shares significant sequence similarity with the yeast choline transporter (CTR gene), exhibiting but limited similarity to the previously reported GABA transporters, i.e., the yeast GAP1 and PUT4 permeases and the rat brain GATs. Induction of UGA4 in the presence of GABA is exerted at the level of UGA4 messenger RNA accumulation, most probably at the level of transcription itself (Andre et al. 1993; Bermudez Moretti et al. 1995).

Arabidopsis proteins capable of transporting GABA were identified by heterologous complementation of a GABA transport-deficient yeast mutant (*gap1*, *put4*, *uga4*) (Breitkreuz et al. 1999). Expression of these *Arabidopsis* transporters allowed the growth of the GABA transport-deficient yeast

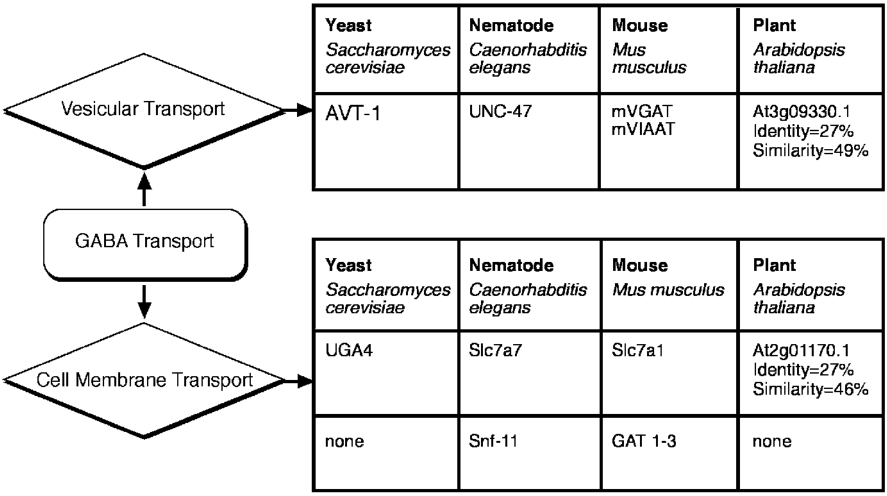

Fig. 12.2. GABA transporters in different organisms. Vesicular and cell-membrane GABA transporters from different organisms are listed in *rows* according to their type and amino acid sequence homologies. Amino acid similarities (%) and identities (%) are presented for *Arabidopsis* proteins in comparison with UNC-47 (vesicular transpoters) and UGA4 (cell membrane transporters)

mutant (*gap1*, *put4*, *uga4*) on GABA as a sole nitrogen source (Breitkreuz et al. 1999). Two plant amino acid transporters, amino acid permease 3 (AAP3) (Fischer et al. 1995) and the proline transporter 2 (ProT2) (Rentsch et al. 1996), effectively mediated GABA transport in yeast. In addition, growth of *Arabidopsis* on a medium supplemented with GABA as the sole nitrogen source provided evidence that plants possess proteins capable of transporting GABA. Recently the subcellular localization and tissue-specific expression of the AtProT protein family was reported (Grallath et al. 2005). Transient expression of green fluorescent protein fused to individual AtProTs in tobacco protoplasts revealed that all three AtProTs were localized at the plasma membrane. Expression and functional analysis in a yeast mutant (*gap1*, *put4*, *uga4*) demonstrated that the affinity of all three AtProTs was highest for glycine betaine, lower for Pro, and lowest for GABA. Also, it has been shown that the expression patterns of the three AtProTs are complementary. AtProT1 expression was found in the phloem, or in phloem parenchyma cells, throughout the plant, indicative of a role in long-distance transport of compatible solutes. In contrast, AtProT2 expression was restricted to the epidermis and to the cortex cells in roots, whereas in leaves expression could be demonstrated only after wounding. AtProT3 expression was restricted to the aboveground parts of the plant and could be localized to the epidermal cells in leaves.

In conclusion, to date, two putative *Arabidopsis* GABA permeases have been characterized (ProT2, AAP3). However, their affinities and specificities for GABA are relatively low. Therefore, other potential plant GABA transporters should be explored. One promising candidate is the *Arabidopsis* homolog of the yeast GABA-specific transporter UGA4 (Fig. 12.2).

12.3 GHB, a By-Product of the GABA Shunt and a Neurotransmitter

12.3.1 From Elixir of Life to Date-Rape Drug

GHB, a by-product of the GABA shunt (Fig. 12.1) has been at the center of public debate since its discovery. Its structure is similar to that of GABA (Fig. 12.1) and it was, for a while, believed to be a wonder cure, and its purported anabolic effect of enhancing body mass prompted its use in the fitness industry (Okun et al. 2001) until 1991, when the US Food and Drug Administration (FDA) removed it from the market. GHB is still in use as an adjunct to anesthesia, and induces a trancelike state that mimics sleep. This property led to its use in sexual violence incidents in after-party hours, where it was mixed in alcoholic beverages. As such, it gained the negative nickname "date rape-drug" (Weir 2000).

GHB is found in all body tissues and, similar to GABA, occurs at high levels in the mammalian brain where it is synthesized and stored in neurons, and is released to the extracellular space upon Ca^{2+}-dependent neuronal depolarization. The mechanisms at the base of its proposed role as an inhibitory neuromodulator within the central nervous system are still being evaluated. GHB has been suggested to be involved in the regulation of GABA, dopamine (Hedou et al. 2000; Howard and Feigenbaum 1997), 5-hydroxytryptamine, acetylcholine, and serotonin metabolism (Gobaille et al. 2002). Moreover its pharmacological actions are thought to be mediated through a receptor pathway mechanism, possibly by modulation of $GABA_B$ receptors (Tunnicliff 1992; Mathivet et al. 1997; Lingenhoehl et al. 1999) as well as through specific GHB receptors (Maitre 1997; Snead 2000; Andriamampandry et al. 2003).

The effects of GHB on animals are diverse. GHB produces deep reversible depression of cerebral metabolism, increases dopamine concentrations, induces hypothermia (Maitre 1997), and decreases stroke volume and heart rate (Mamelak 1989). A protective mechanism mediated by a GHB-induced trancelike state has been proposed in cases of injury and hypoxia conditions

(Mamelak 1989, 1997; Boyd et al. 1990; Kolin et al. 1993). French scientist and philosopher Laborit reported on anticonvulsive effects of GHB (Laborit 1964). Laborit (1973) suggested that GHB causes the nervous system to catabolize glucose mainly through the pentose shunt in glial cells, thus utilizing less oxygen, with consequential decrease in reactive oxygen intermediates (ROI), and the production of reducing power to counteract the high daytime glycolytic-mitochondrial metabolic activity (Genova et al. 2004).

12.3.2 SSADH Inborn Deficiency: the Dark Side of GHB

In the past 20 years, interest has grown toward GHB in connection to a rare inborn error of GABA catabolism in humans due to SSADH deficiency, an autosomal-recessive inherited disorder, of which there are likely less than 400 patients worldwide (Gupta et al. 2003). This pathology, which manifests physiologically as GHB–aciduria, has at least two neuroactive species, GABA and GHB. The understanding of the possible mechanisms behind this pathology has been aided by the study of murine knockout models. The complete absence of SSADH enzyme activity in neuronal and peripheral tissue leads to the birth of *ssadh* mice characterized by a phenotype reminiscent of the human disease (Gupta et al. 2003). The pathological characters include neurological impairment and growth retardation, ataxia, and seizures, which eventually lead to 100%mortality, in addition to a severe GHB accumulation (35–40-fold) and a minor increase in GABA (2–3-fold) in the mice urine, brain, and peripheral brain extracts. An intriguing reduction of glutamine was also reported. In contrast, other metabolites linked to the GABA shunt, among them glutamate, the precursor of GABA synthesis, and intermediates of TCA cycle, have not shown significant changes (Hogema et al. 2001; Gibson et al. 2002).

The high accumulation of GHB raises questions on the efficiency of its catabolism. The oxidation of GHB to SSA is a rate-limiting step, proceeding at approximately 1,000th of the rate at which SSA is oxidized to succinate by SSADH (Kaufman and Nelson 1991). In mammals two enzymes are thought to be responsible for the catabolism of GHB to SSA: (1) Schaller et al. (1999) suggest a role for SSA reductase/AFAR (also referred to as GHBDH) in the reversible conversion of SSA to GHB; (2) Kaufman and Nelson (1991) have shown that aldehyde reductase (glucuronate reductase or l-hexonate dehydrogenase, EC 1.1.1.19, in their nomenclature) can oxidize GHB in unison with the reduction of glucuronate.

12.3.3 The GABA Shunt and Redox Imbalance: from Bacteria to Humans

As indicated earlier, some of the effects of GHB in mammals suggest its involvement in maintaining the redox state of cells. Studies in microorganisms (Poole and Allaway 2000) also suggested the GABA shunt as part of a metabolic network that helps maintain redox equilibrium. In anoxic environments bacteria of the orders *Clostridiales* (*Firmicutes*, phylum *Bacteria* XIII) and *Fusobacteriales* (*Fusobacteria*, phylum *Bacteria* XXI; Garrity 2001) ferment amino acids in the anaerobic food chain, by which proteins and other polymers are degraded to methane and CO_2. Bacterial anaerobic catabolism of glutamate to fatty acids involves the decarboxylation of glutamate to GABA and its conversion via GHB to acetate and butyrate (Buckel 2001). In legume nodules glutamate appears in large quantities (Miller et al. 1991; Vance and Heichel 1991; Salminen and Streeter 1992). It was shown that this amino acid accumulates partly following the inhibition of the bacterial enzyme 2-oxoglutarate dehydrogenase (2OGDH) during symbiosis (Walshaw et al. 1997), when high NADH levels are found in bacteroids, owing to oxygen limitation. Thus, the removal of intermediates, reductants, or ATP from the bacteroids is imperative for TCA cycle efficiency (Lodwig and Poole 2003). Under hypoxic conditions the GABA shunt may bypass the 2OGDH block and feed the TCA cycle with succinate or remove two NADH molecules from the system by producing GHB (Fig. 12.1; Miller et al. 1991; Prell et al. 2002). In turn, GHB may be used as a precursor for the synthesis of the carbon-sink polymer polyhydroxybutyrate (PHB). PHB also accumulates during the stationary phase of growth of bacteroids when cells become limited for an essential nutrient but have excess carbon source (Udvardi and Day 1997; Poole and Allaway 2000).

In mammal nerve cells, the metabolic processes under hypoxic conditions are still under debate. Tretter and Adam-Vizi (2000) tested the relation between ROI level and enzymes of the TCA cycle in nerve terminals. Their study revealed that, similarly to bacteroids, 2-OGDH is a rate-limiting enzymes of the TCA cycle, and as such it may have a flux-controlling function (Tretter and Adam-Vizi 2000, and reference therein). Inhibition of 2OGDH plays a critical role in limiting the amount of reducing power in the form of NADH during H_2O_2-induced oxidative stress (Chinopoulos et al. 1999; Tretter and Adam-Vizi 2000). H_2O_2 modulates 2-OGDH activity possibly by modifying sulfhydryl group(s) on 2OGDH (Nulton-Persson et al. 2003). The GABA shunt may function as a route to overcome the 2OGDH block and further provide the system with NADH during the NAD^+-dependent SSADH-driven reaction.

These and other pieces of evidence suggest the GABA shunt may be of significance in maintaining redox equilibrium. A deficient shunt and

concomitant overproduction of GHB may alter cell function by impairing its redox state (Gupta et al. 2003; Visser et al. 2002; Nakamura et al. 2001) or interfere with mitochondrial metabolism (Taberner et al. 1972; Godin et al. 1969), which might in turn lead to the amplification of oxidative stress (Genova et al. 2004).

12.3.4 The GABA Shunt, GHB, and the Redox State in Plants

A recent study in *Arabidopsis* showed that SSADH deficiency leads to detrimental effects on plant development under stress conditions (Bouché et al. 2003). The light-fluence-dependent appearance of necrotic lesions on leaves, and the accumulation of ROI in SSADH-deficient plants, particularly under high-fluence light, or in response to other stresses (e.g., heat shock) suggested a redox related function of the GABA shunt in plants (Bouché et al. 2003). Moreover, under conditions where SSADH is not functional, there might be a toxic accumulation of by-products of the TCA cycle and/or the GABA shunt. Similar to the situation in mammals, GHB might accumulate under these conditions, and cause tissue toxicity. Recently, an *Arabidopsis* GHBDH cDNA was identified and characterized by functional complementation of an SSADH-deficient yeast mutant (Breitkreuz et al. 2003). The encoded enzyme synthesizes GHB from its precursor SSA. Recent evidence also showed the accumulation of GHB in plants in response to hypoxic conditions (Allan et al. 2003; Breitkreuz et al. 2003). Earlier, Busch and Fromm (1999) showed that *Arabidopsis* SSADH is negatively regulated by NADH to NAD^+ ratios as well as by adenylate composition, AMP:ADP:ATP. Alterations in these metabolite ratios are characteristic of hypoxic condition (Wigge et al. 1993; Shelp et al. 1995). Therefore, we may speculate that a decrease in SSADH activity would boost GHB production as a consequence of the accumulation of the common unstable precursor SSA (Fig. 12.1). Moreover, similar to bacteria, increase in NADH/ROI in hypoxic cells would inhibit the 2-OGDH, leading to redirection of Glu and 2OG through the GABA shunt.

The *ssadh* mutant in *Arabidopsis* provided an opportunity for further studies of the physiological role of the GABA shunt and GHB in plants. In a recent pharmacological and metabolic study on *Arabidopsis ssadh* mutants, Fait et al. (2005) showed that GHB is abundant in the *ssadh* mutant. High-fluence light further increased GHB content in the mutant, which was associated with ROI accumulation and the appearance of lesions on the leaves. These observations suggested that (1) the increased level of GHB in the mutant is likely caused by the metabolic block resulting from

the SSADH deficiency and the accumulation of SSA (Fig. 12.1) and (12.2) GHB levels might lead to redox imbalance and tissue toxicity. To test the latter hypothesis the authors aimed at reducing GHB levels utilizing vinyl-GABA (Vigabatrin; VGB), a specific inhibitor of GABA-T (Fig. 12.1; Lewis and Wallace 2001). Indeed, VGB was found effective in reducing the levels of GHB and the symptoms of SSADH deficiency, like it does in animals (Hogema et al. 2001; Gupta et al. 2002). Moreover, VGB prevented the accumulation of H_2O_2 in the *ssadh* mutant (Fait et al. 2005). Thus, GHB is tightly linked to ROI levels in plants.

12.4 Conclusions and Future Perspectives

Recent studies reinforce the notion that the neurotransmitters GABA and GHB may function as signaling molecules in plants, playing a role in modulating carbon:nitrogen metabolism, energy balance, and redox equilibrium. Hence, not surprisingly, these two neurotransmitters are associated with stress responses in plants, as the levels of both change dramatically in response to stress situations. Moreover, like in animals, GABA seems to play a role in cell guidance. However, the mode of action of GABA and GHB in plants is still unknown. Therefore, an effort to identify receptors of plant neurotransmitters, as well as their intracellular and intercellular transporters is crucial toward deciphering their functions and mode of action. Future studies should also attempt to investigate in vivo real-time dynamics and spatial distribution of neurotransmitters within plant cells and throughout the plant under different physiological situations. These cellular studies with the help of functional genomic tools (e.g., mutants) and other whole-genome approaches are likely to unravel fascinating mechanisms of neurotransmitter signaling in plants.

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13 The *Arabidopsis thaliana* Glutamate-like Receptor Family (*At*GLR)

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Abstract The 20 genes that encode the *Arabidopsis thaliana* glutamate-like receptor family (*At*GLR) share significant similarity in amino acid coding sequence and predicted secondary structure with animal ionotropic glutamate receptor (iGluR) subunits. In animals, iGluR subunits form glutamate-gated non-selective cation channels (NSCCs) catalysing Na^+ and/or Ca^{2+} influx into cells; in one iGluR subfamily glycine also is required as a coagonist. In *Arabidopsis*, both glutamate and glycine have been demonstrated to depolarise the plasma membrane and increase $[Ca^{2+}]_{cyt}$, and iGluR antagonists blocked these effects. *At*GLRs are therefore predicted to function in an analogous manner to iGluR. Attempts to functionally characterise *At*GLRs in heterologous expression systems have proved inconclusive with no ligand-gated activity detected. Research into the glutamate receptor-like family has been hindered by the lack of phenotypes associated with the *At*GLR genes but several phenotypes associated with *At*GLR overexpression and knockout have recently given hints as to their function. *At*GLR have been implicated in light and C:N signalling, hypocotyl detiolation, root growth, abscisic acid (ABA) metabolism, stress responses, and general ion transport. This review will concentrate on recent developments in the *At*GLR field, including the roles and effects of glutamate and glycine and related metabolites in plant physiology relative to potential roles for *At*GLRs. It will examine progress made toward defining the functions of particular *At*GLRs and will conclude by recommending potentially fruitful avenues of future research.

13.1 Introduction

Twenty genes in the *Arabidopsis thaliana* genome encode subunits of glutamate-like receptors (*At*GLRs) (Lam et al. 1998; Lacombe 2001a). *At*GLR subunits are so named owing to their similarity in amino acid coding sequence, and predicted secondary structure, to animal ionotropic glutamate receptor (iGluR) subunits (Lam et al. 1998; Fig. 13.1). Phylogenetic analysis suggests that the evolution of the *At*GLR subunits predates the divergence of plants and animals (Chiu et al. 1999, 2002). It is believed that their evolutionary precursor evolved from the insertion of an inverted K^+-selective ion channel into an amino acid binding protein (Wo and Oswald 1995). The discovery of a functional glutamate-activated K^+-selective channel (GluR0) in the prokaryotic cyanobacteria *Synechocystis* provides compelling support for both hypotheses (Chen et al. 1999). To date, the functional roles of *At*GLR subunits have not been clearly defined.

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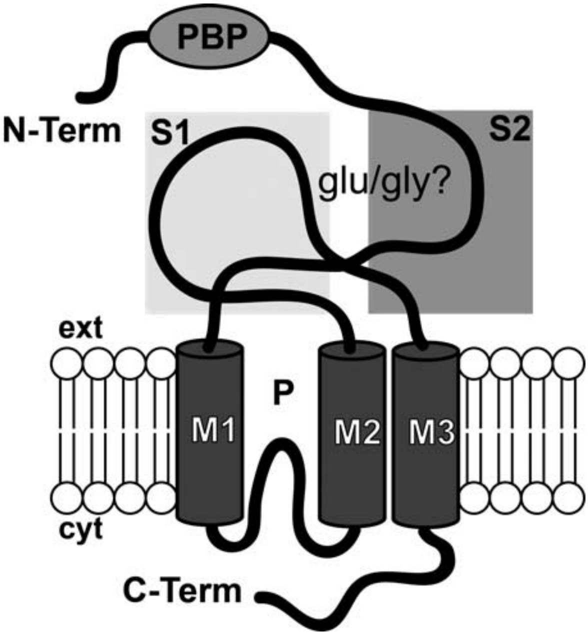

Fig. 13.1. *Arabidopsis thaliana* glutamate-like receptors (*At*GLRs) are predicted to share the ionotropic glutamate receptor (iGluR) channel structure, with three transmembrane domains (*M*1, *M*2, *M*3) and a *P* domain forming a membrane-embedded pore loop when subunits combine into a functional tetramer (Colquhoun and Sivilotti 2004). *At*GLRs also share with iGluRs a similar putative ligand-binding motif formed by the interactions of the S1 and S2 domains. Most *At*GLRs sequence before the *S*1 and after *S*2, including M3, which is absent in *Synechocystis* GluR0, show limited sequence similarity to iGluR (Chiu et al. 2002). Both *At*GLR and iGluR have long N-terminal domains (N-term) with similarity to bacterial periplasmic binding proteins (PBP); the role of the N-terminus is not clearly defined but it is thought to be involved in targeting, translocation and degradation (Kato et al. 2005). P is confusingly sometimes referred to as M2; therefore, M2 and M3 domains are then named M3 and M4, respectively; S1 and S2 have been called GlnH1 and GlnH2

In animals, iGluR subunits are generally non-selective cation channels (NSCCs) that propagate impulses across vertebrate neuronal or invertebrate neuromuscular junctions through glutamate-gated Na^+ and/or Ca^{2+} entry (Dingledene et al. 1999). iGluR subunits can be grouped into seven subfamilies according to primary amino acid sequence which have been further classified into four receptor families according to their sensitivity to specific amino acids and electrophysiological as well as pharmacological properties [GluRα (GluR1–4); α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, AMPA], [GluRβ (GluR5–7), GluRγ (KA1,2); kainate], [GluRδ (δ1,2); orphan], [GluRε (NR2A–D), GluRζ (NR1), GluRχ (NR3A,B); *N*-methyl-D-aspartate, NMDA] (Hollmann and Heinemann 1994; Sprengel

and Seeburg 1995; Dingledene et al. 1999). In addition to glutamate, NMDA receptors require glycine as a coagonist (Dingledine et al. 1999). *At*GLRs have been divided into three clades according to sequence (*At*GLR1.1–1.4; 2.1–2.9; 3.1–3.7) (Lacombe et al. 2001a; Chiu et al. 2002).

This brief review will concentrate on recent developments in the field, specifically exploring the roles and effects of glutamate and glycine, and related metabolites, in plant physiology relative to potential roles for *At*GLRs. It will then examine the progress made toward defining the functions of particular *At*GLRs and will conclude by recommending potentially fruitful future avenues of research.

13.2 Roles (and Effects) of Glutamate, Glycine and Interrelated Amino Acids in Plants

13.2.1 Effects of Amino Acids on Plant Development

Plant development is sensitive both to light and to tissue C:N, which determines the allocation of biomass between root and shoot (Thum et al. 2003). The molecules that signal this ratio, and the receptors that detect it, are largely unknown, but it is thought that certain amino acids signal nitrogen status (Thum et al. 2003). Attempts to relate *At*GLRs to C:N signalling have shown that iGluR agonists and inhibitors affect hypocotyl development. 6,7-Dinotroquinoxaline 2,3-(1*H*,4*H*)-dione (DNQX) caused an etiolated phenotype in seedlings grown in light but not those grown in the dark (Lam et al. 1998) and this was reversed by glutamate and/or glycine (Dubos et al. 2003). *S*(+)-β-Methyl-α,β-diaminopropionic acid (BMAA) also caused etiolation in light but inhibited hypocotyl elongation in the dark, and both effects were reduced by glutamate and glutamine (Brenner et al. 2000).

Rhizosphere amino acids may signal the location of nutrient-rich organic matter (Filleur et al. 2005). Both glutamate and glycine have been demonstrated to modify root elongation. Micromolar levels of glutamate induced root bending toward a glutamate source, whereas greater concentrations inhibited primary root growth (Filleur et al. 2005). Glycine has also been demonstrated to exert positive (White 1939; Fries 1953; Skinner and Street 1953) and negative (Skinner and Street 1953) effects on root growth. Amino acids also function in developmental and stress responses both as signals and as osmoprotectants. For instance, the glutamate-derived γ-aminobutyric acid (GABA) and glycine-derived glycine betaine function

as compatible solutes under salt and water stress in *Arabidopsis* (Chen and Murata 2002; Kaplan et al. 2004). GABA is a key neurotransmitter in animals and is synthesised rapidly in plants in response to biotic and abiotic stresses, including anoxia, oxidative stress, and mechanical stress (Scott-Taggart et al. 1999). Changes in GABA levels are also implicated in plant development, and a gradient of GABA directs pollen tube formation (Palanivelu et al. 2003). β-Aminobutyric acid (BABA) potentiates plant defences against pathogen attack (Zimmerli et al. 2000). Glycine is also involved in the movement response in *Mimosa pulvinus* cells (Otsiogo-Oyabi and Roblin 1985).

13.2.2 Glutamate and Glycine as Signalling Molecules

Recent evidence supports the hypothesis that glutamate and glycine function as signalling molecules in *Arabidopsis* (Dubos et al. 2003). Aside from their obvious effects on plant growth and development glutamate can induce an atypical depolarisation of the electrical potential gradient across the plasma membrane (*Em*) of *Arabidopis* root, hypocotyl, and leaf mesophyll cells when compared with other amino acids (Dennison and Spalding 2000; Sivaguru et al. 2003; Meyerhoff et al. 2005) and new data suggest that this occurs with glycine as well (Qi et al. 2004; M. Gilliham, unpublished). The depolarisation is multiphasic, consisting of an initial rapid, substantial transient with duration in the order of tens of seconds followed by a smaller secondary phase that plateaus until removal of the agonist. The secondary phase seen in the presence of other amino acids probably reflects the activity of proton-coupled amino acid symporters (Kinraide and Etherton 1980; de Jong and Borstlap 2000), whereas the primary transient is probably due, at least in part, to the passage of Ca^{2+} across the plasma membrane. The primary depolarisation by glutamate is, at least initially, coincidental with a transient rise in $[Ca^{2+}]_{cyt}$, as indicated by whole seedlings constitutively expressing the chemiluminescent Ca^{2+}-binding protein aequorin, with both phenomena being abolished by the non-specific calciumchannel blocker La^{3+} (Dennison and Spalding 2000; Sivaguru et al. 2003; Meyerhoff et al. 2005).

Both the glutamate-induced primary *Em* transient and the rise in $[Ca^{2+}]_{cyt}$ appear to be concentration-dependent, first detectable around 50 µM and saturate in the low millimolar range (Demidchik et al. 2004; Meyerhoff et al. 2005). As has been observed in animals, glutamate and glycine applied together synergistically decrease the amount of agonist needed to increase $[Ca^{2+}]_{cyt}$ to 10 µM (Dubos et al. 2003). Subsequent applications of agonist decrease both these responses (Meyerhoff et al. 2005; Qi et al. 2005; M.

Gilliham, unpublished), possibly indicating a desensitisation of response (Everts, et al. 1999; Sun et al. 2002). Unpublished results of Qi et al (2005) suggest that glutamate desensitised the response to glycine in the hypocotyl, but not vice versa, whereas no such specificity in desensitisation response has been observed in roots (M. Gilliham, unpublished). De novo synthesis of proteins may be required for full recovery of the response, since recovery is inhibited by cycloheximide (Meyerhoff et al. 2005).

Neither response is initiated by GABA, NMDA, AMPA or KA, supporting the divergence of *At*GLR and iGluR before they developed unique ligand specificity (Chiu et al. 1999). However, DNQX and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) can abolish the glutamate-induced and/or the glycine-induced $[Ca^{2+}]_{cyt}$ increase (Dubos et al. 2003; Meyerhoff et al. 2005). The cold-shock-induced rise in $[Ca^{2+}]_{cyt}$ is unaffected by DNQX (or previous application of glutamate) (Dubos et al. 2003), indicating separate Ca^{2+} response pathways but also specificity in the CNQX/DNQX antagonism. The NMDA/glutamate site binding iGluR antagonist AP-5 also blocks both *Em* depolarisation and the Ca^{2+}-dependent microtubule depolymerisation induced by glutamate (Sivaguru et al. 2003), however, its effect on $[Ca^{2+}]_{cyt}$ has not been measured directly. Interestingly NMDA also depolymerises microtubules despite not inducing a $[Ca^{2+}]_{cyt}$ or an *Em* response (Sivaguru et al. 2003), suggesting that caution must be exercised when using iGluR agonists/antagonists as indication of *At*GLR involvement in these processes. It should also be noted that NMDA-type iGluRs, the only iGluR subtype to bind glycine, are not inhibited by CNQX/DNQX which bind at the glutamate binding site of AMPA/KA iGluR.

L-glutamate also induced the influx of Na^+ and Ca^{2+} in a small percentage of patch-clamped protoplasts derived from *Arabidopsis* root cells by activating ‘spiky’ inward currents at hyperpolarised potentials with external Ca^{2+} or less erratic currents with Na^+ (Demidchik et al. 2004). Glycine can also activate an instantaneous current in patch-clamped protoplasts derived from wheat root cells (M. Gilliham, unpublished). The effect of glutamate on unidirectional influx of Na^+ into *Arabidopsis* roots was inconclusive (Demidchik et al. 2004). Because Na^+ influx is mainly via NSCCs, which are permeable to other monovalent cations, the movement of Na^+, K^+, Cs^+, and NH_4^+, although not dependent upon a glutamate-induced depolarisation may contribute to it.

Sivaguru et al. (2003) have suggested that *At*GLRs are involved in root responses to toxic Al^{3+}. They propose that Al^{3+}, known to induce organic anion release in some species (Ma and Furukawa 2003), stimulates glutamate release, which activates Ca^{2+} influx via plasma-membrane-localised *At*GLR, leading to *Em* depolarisation and microtubule depolymerisation. They observed that 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), known to inhibit the Al^{3+}-activated organic anion channel of the root

apex (Zhang et al. 2001), blocks Al^{3+}-induced *Em* depolarisation and Ca^{2+}-dependent microtubule depolymerisation but does not block these in response to L-glutamate. An alternative sequence of events in response to glutamate is suggested by plant action potentials. The action potential of giant algal cells is dependent on three phases: (1) an initial small Ca^{2+} influx that (2) stimulates a substantial Cl^- efflux to below E_K which (3) stimulates an efflux of K^+ repolarising *Em* (Shepherd et al. 2002). It is entirely possible that the depolarisation induced by glutamate could involve a similar chain of events. Qi et al. (2005) have reported that, in contrast to lone applications, a combination of NPPB and 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (a Ca^{2+} chelator) can decrease the glutamate-induced *Em* depolarisation, supporting the hypothesis that ligand treatment affects membrane permeability to multiple ions. Taken together with the putative role of *At*GLR in de-etiolation it is tempting to suggest an involvement of *At*GLR in the blue-light induced *Em* depolarisation of the hypocotyl (Cho and Spalding 1996).

13.3 Roles of AtGLR

13.3.1 Expression

Individual *At*GLRs show complex spatio-temporal expression patterns during plant development and a diversity of responses to stresses (Sect. 13.3.5). Data from multiple sources (RT-PCR, promoter:reporter fusions, expressed sequence tags, microarrays) indicate widespread *AtGLR* transcription, typically with low messenger RNA (mRNA) abundance. RT-PCR indicated that most *AtGLR*s were transcribed in root, shoot, flower, and silique in 8-week-old plants, although transcripts of several clade 2 genes (2.1–2.3, 2.9) were detected roots only (Chiu et al. 2002). At the single-cell level, individual mature leaf epidermal and mesophyll cells contained three to nine *At*GLR transcripts, with no consistent pattern except that *AtGLR3.7* was ubiquitous (Roy et al. 2004). The putative promoter of *AtGLR3.7* was fused to the β-glucuronidase (GUS) reporter and indicated widespread transcription in all tissues in seedlings and in root vasculature, leaves, siliques (but not seeds), and flowers of mature plants (Essah 2002). The near ubiquity of *AtGLR3.7* transcripts raises the possibility that *AtGLR3.7* could be a key subunit involved in a variety of *At*GLR heteromeric proteins, analogous to NMDAR1 amongst NMDA receptors. Other *AtGLR*s may be more confined in their expression, for instance, localisation of *AtGLR3.2* by promoter:GUS fusion, RT-PCR, and an antibody raised to the

C-terminus indicated strongest expression in developing floral stems and vasculature (Kim et al. 2001; Turano et al. 2002). A number of *At*GLRs show high expression in senescent leaves, including all *At*GLR1, *At*GLR*2.5*, *At*GLR2.7–9, and *At*GLR3.4 (Zimmermann et al. 2004; Meyerhoff et al. 2005). Promoter:GUS fusions for *AtGLR2.8*, *AtGLR2.9*, and *AtGLR3.4* have indicated their expression throughout the leaf mesophyll but are greatest surrounding vascular bundles as well as hydathodes, suggestive of a role in metabolite re-mobilisation during senescence (O. Meyerhoff and D. Becker, unpublished).

Most *At*GLRs have a hydrophobic N-terminus predicted to be cleaved after targeting the nascent protein to the endoplasmic reticulum for processing (Davenport 2002). No clear picture has yet emerged concerning the subcellular distribution of individual plant glutamate receptors. However, using green fluorescent protein (GFP) or GUS–GFP tags several *At*GLR (*At*GLR3.4, 3.7, and a radish GLR) have been localised to the plasma membrane of onion epidermis cells, *Arabidopsis* and tobacco protoplasts (D. Becker, unpublished; R. Davenport, unpublished; Kang and An 2003). However, these studies should be interpreted with caution since incorporation of tags and/or overexpression can cause mistargeting (*At*GLR3.4 has also been reported to be expressed in plastids) (Szabo et al. 2004). An antibody to the C-terminus localised *At*GLR3.2 to the membrane fraction but was not used to distinguish the membrane involved (Turano et al. 2002).

13.3.2
Amino Acid Binding and *At*GLR Regulation

*AtGLR*s have been predicted to function in an analogous manner to iGluRs. Phylogenetic analysis predicts that *At*GLRs share greatest sequence similarity with NMDA receptors despite sharing more invariant residues with GluRδ receptors (Chiu et al. 1999, 2002). Subunit–ligand interactions for *At*GLR were modelled by superimposing aligned sequences onto the ligand-binding domains of a rat iGLRα crystal structure (Dubos et al. 2003). The docking algorithm OXDOCK (Glick et al. 2002) found that only *At*GLR1.1 would be predicted to bind glutamate, whereas the remaining 19 predicted *At*GLR subunits would not owing to steric hindrance in the ligand-binding site (Dubos et al. 2003). Instead, these 19 subunits share a high degree of similarity with glycine-binding domains, and are predicted to bind glycine. It is intriguing that *Arabidopsis* possesses a greater number of *At*GLR subunits that are predicted to bind glycine, as opposed to glutamate, and may suggest that glycine plays a more important role in signalling than had previously been suspected.

13.3.3 Are *At*GLRs Ion Channels?

*At*GLRs are thought to encode subunits of plant NSCCs (Davenport 2002; Demidchik et al. 2002; Demidchik, this volume). However, despite profound amino acid sequence similarity with iGluRs the residues within the putative pore region of *At*GLRs are unlike any other ion channel known (Davenport 2002). This makes predictions about their ion selectivity, if they are indeed channels, impossible. Furthermore, although *At*GLRs are divided into three clear clades upon the sequence similarity of their whole sequence if only the M1, P, and M2 domains are compared then the similarity in and between clades breaks down. This may indicate either a loss of channel function or similar selectivity and/or gating properties between *At*GLR clades, in contrast to iGluR subfamilies (Chiu et al. 2002).

Attempts to functionally characterise *At*GLRs in heterologous expression systems have so far proved inconclusive. The expression of several group 2 members in *Xenopus* oocytes did not result in any detectable current, even following fusion with the signal peptide from the rat GluR6 which facilitated the functional characterisation of the previously non-functional *Synechocystis* GluR0 (Lacombe et al. 2001b; B.R. Davies and R. Davenport, unpublished). The injection of *AtGLR3.4* and *AtGLR3.7* complementary RNA (cRNA) results in the activation of cation-permeable conductances at the oocyte plasma membrane and the consecutive activation of the intrinsic Ca^{2+}-activated chloride current (ICl_{Ca}) (D. Becker, unpublished; Cheffings 2001; Gilliham 2005, unpublished; Lacombe et al. 2001b). Activation of the ICl_{Ca} does indicate that $[Ca^{2+}]_{cyt}$ has been elevated but it is not proof that the injected cRNA forms a channel that catalyses the influx of Ca^{2+} across the plasma membrane. ICl_{Ca} activation may be the sole result of upregulation of additional endogenous channels in intracellular compartments or the plasma membrane such as Ca^{2+}-permeable hyperpolarisation-activated cation channels (Tzounopoulos 1995; Kuruma et al. 2000) or the disruption of cellular homeostasis (Weber 1999). Cheffings (2001) has suggested that *At*GLR3.7 is constitutively active and catalyses the voltage-independent movement of Na^+, K^+, and Ca^{2+} across the plasma membrane when expressed in *Xenopus* oocytes. Injection of the *At*GLR3. 7 coding sequence containing stop codons reduced the instantaneous current component at all voltages to wild-type levels, indicating that *At*GLR3. 7 may encode a channel. The possibility of constitutive *At*GLR channel activity is supported by the sequence of *At*GLR M2 domains, which resemble sequences known to induce constitutive activity in some iGluRs (Chiu et al. 1999; Yuzaki 2003).

Disappointingly, no currents have been activated by L-glutamate, glycine, or a combination of both, at any voltage, at various pH_{ext}, with a range of external cations, in *Xenopus* oocytes at levels over control following the

injection of any *At*GLRs. Additionally, ABA, BMAA, GABA, NMDA, and glutamine have not activated any currents in any heterologous system containing any AtGLRs as tested so far (Cheffings 2001; Lacombe et al. 2001b; Meyerhoff et al. 2005; M. Gilliham, unpublished), although incubation of the *At*GLR3. 4 injected oocytes with DNQX did reduce the Na^+-permeable "leak" current (M. Gilliham, unpublished).

Although at least *At*GLR3.4 is expressed at the plasma membrane in heterologous systems (D. Becker, unpublished), it is possible that *At*GLRs may require plant-specific processing for proper function. It is possible that the activity of some *At*GLRs is modified by endogenous subunits in oocytes and other heterologous systems, resulting in activation of atypical currents or loss of *At*GLR channel function (Green et al. 2002; Anantharam et al. 2003). Other proteins or cofactors may also be needed for proper activity of *At*GLR in heterologous systems, for instance, SOL-1, a CUB-like protein, colocalises at the neuron cell surface with GLR1 in *Caenorhabditis elegans* and is required for ligand-gated GLR1 activity (Zheng et al. 2004).

In planta evidence for ion channel activity of glutamate receptors is limited. Kim et al. (2001) constitutively overexpressed *At*GLR3. 2, which led to signs of Ca^{2+} deficiency and a hypersensitivity to K^+ and Na^+. Although total tissue Ca^{2+} was the same in wild-type plants and plants overexpressing *At*GLR3.2, medium supplemented with Ca^{2+} could rescue the mutant phenotype. On the basis of these findings *At*GLR3.2, which is most abundantly expressed in vascular tissues, has a proposed role in Ca^{2+} distribution within the plant (Kim et al. 2001; Turano et al. 2002). Antisense *At*GLR1.1 plants did not show hypersensitivity to K^+ and Na^+ compared with wild-type plants, but root growth was more inhibited by high levels of Ca^{2+} (Kang and Turano 2003). In unpublished work, Qi et al. (2004) have reported that *AtGLR* T-DNA insertional mutants have aberrant growth rate responses to glutamate or light. Knockouts of *AtGLR3.3* also displayed reduced depolarisations in the root and hypocotyl associated with glutamate or glycine, whereas knockouts of *AtGLR3.4* showed reduced response to glycine only (Qi et al. 2005).

13.3.4 C:N Signalling

Phenotypic analysis of antisense *At*GLR1.1 (*antiAtGLR1.1*) plants has implicated a role for *At*GLR1.1 in C:N perception and signalling (Kang and Turano 2003; Kang et al. 2004). *antiAtGLR1.1* seed germination was inhibited by sucrose, but rescued by supplemental nitrate. Compared with wild-type, seed germination of *antiAtGLR1.1* plants was also more susceptible to inhibition by DNQX, possibly because *antiAtGLR1.1* suppressed *At*GLR activity

sufficiently to make plants more susceptible to DNQX. BMAA counteracted the inhibitory effect of DNQX on *antiAtGLR1.1* seed germination despite previous observations that DNQX and BMAA have superficially analogous effects on plant growth and development (Brenner et al. 2000; Sect. 13.2.1), suggesting one must be cautious in ascribing a precise mode of action to either of these compounds in plants. Glutamate (and to a lesser extent glutamine) rescued germination of *antiAtGLR1.1* seed by DNQX, but both were less effective than BMAA. It was proposed that glutamate uptake or distribution was poor compared with DNQX, or that glutamate is not the natural ligand for *At*GLRs. However, as *AtGLR1.1* encodes the only subunit predicted to bind glutamate (Dubos et al. 2003) *antiAtGLR1.1* plants would be expected to be particularly impaired in glutamate perception.

The expression of genes and the accumulation of proteins related to carbon and nitrogen metabolism, as well as ABA responses and metabolism, were altered in *antiAtGLR1.1* plants (Kang and Turano 2003; Kang et al. 2004). For instance, *antiAtGLR1.1* plants had elevated ABA levels, increased transcript abundance of ABA biosynthetic genes, enhanced ABA sensitivity, reduced stomatal apertures, and increased drought resistance. Given that ABA has been implicated in sugar signalling (Gibson 2005), the alteration of ABA metabolism and response in *antiAtGLR1.1* plants provides another link to carbon sensing in these plants.

A model has been proposed that places *At*GLR1.1 at the interface of sucrose and amino acid signalling, suggesting *At*GLR1.1 is stimulated by amino acids and suppresses ABA biosynthesis and sugar signalling, allowing seed germination to occur (Kang and Turano 2003; Kang et al. 2004). The model also suggests that *At*GLR1.1 is inhibited by sucrose, which, in turn, allows sugar signalling and ABA biosynthesis to occur and results in inhibition of seed germination. However, the strict involvement of *At*GLR1.1 in C:N signalling needs to be confirmed. The segment of the *At*GLR1.1 gene that was used to generate the antisense construct shares sequence identity with several *At*GLR genes, including *AtGLR*1.2, *AtGLR*1.4, and *AtGLR*3.6. It may be that these other members of the *AtGLR* family were also suppressed by the antisense construct and therefore they may also contribute to the phenomena observed in the *antiAtGLR1.1* lines.

13.3.5 Stress Responses

The GENEVESTIGATOR microarray toolbox suggests that, with respect to abiotic stress, there is no single stress factor or plant hormone that induces or represses expression of *At*GLR in general (Zimmermann et al. 2004). For instance, while *AtGLR*2.5 and *AtGLR*3.2 were upregulated by ABA,

*AtGLR*2.1 and *AtGLR*3.5 were downregulated. *At*GLR1.3 and *AtGLR*1.1 seemed not to respond at all to this stress hormone despite the latter being implicated in the regulation of ABA biosynthesis and *antiAtGLR1.1* plants exhibiting elevated ABA levels (Kang and Turano 2003; Sect. 3.3). Maathuis et al. (2003) demonstrated that members of the *AtGLR* family showed time-dependent changes in transcription in response to Ca^{2+} deprivation and excess Na^{+}, but not K^{+} deprivation, supporting the prediction that they function as NSCCs.

Analysis of GUS expression under control of the *AtGLR3.4* promoter suggested that *At*GLR3.4 expression was induced by mechanical stress such as wounding or touch and cold (Meyerhoff et al. 2005). The upregulation of *AtGLR3.4* transcription by wounding and during senescence could suggest an involvement of methyljasmonate (Sect. 13.3.1), a common component of both processes (Devoto and Turner 2005). Quantitative RT-PCR analyses of *At*GLR3.4 expression revealed that cold-induced transcription was fast, reaching peak transcript levels after 30 min. Additionally, La^{3+} inhibited cold-induced *AtGLR3.4* transcription, suggesting the involvement of Ca^{2+}. *AtGLR1.2* and *AtGLR2.5* are also upregulated by cold treatment (Zimmermann et al. 2004).

13.4 Conclusions and Future Perspectives

Research into the glutamate receptor-like family has been hindered by the lack of phenotypes associated with the *At*GLR genes. The novel pore region of the predicted proteins and the paucity of understanding of NSCC function in vivo have made it difficult to predict function. However there are now several phenotypes associated with *At*GLR overexpression (Kim et al. 2001) and knockout (Kang and Turano 2003; Kang et al. 2004), and systematic electrophysiological characterisation of single *At*GLR knockout plants is underway (Qi et al. 2004, 2005; Gilliham 2005). Despite recent progress, several fundamental questions still remain regarding *At*GLR function.

13.4.1 Expression

One promising approach to predicting *At*GLR function is the study of *At*GLR gene expression in response to developmental and environmental cues. In particular, very rapid changes in gene expression in response to environmental signals (such as application of a ligand, or stresses) implicate the gene product in specific responses to stimuli (Meyerhoff et al. 2004,

2005). It is also desirable to follow changes in abundance at the protein level (to substantiate, for instance, the possibility of rapid protein turnover in response to stresses), and to determine the membrane localisation of *At*GLR with confidence. GFP tags can interfere with normal protein targeting, especially in the case of endomembrane proteins where both the N- and the C-termini as well as internal signals may be involved in targeting, and it is desirable to have a test of retention of functionality or phenotype of the tagged protein (which we do not have at present for most *At*GLRs). Alternatives to large tags such as GFP include antibodies or smaller tags (which could be incorporated internally in the *At*GLR protein to allow in vivo monitoring of membrane protein dynamics as in the case of iGluR tagged with a toxin binding site; Sekine-Aizawa and Huganir 2004).

13.4.2 Ligand Binding and Regulation

The ligand-binding profile of *At*GLR needs to be examined experimentally. Although glutamate and glycine binding has been modelled, no ligand-binding assays have been performed in vitro to confirm the prediction of the model. It is possible that other ligands exist for *At*GLR and elucidation of these could inform phenotyping studies (Sect. 13.4.3). In the absence of a functional expression system, ligand binding could be explored by constructing chimaeras consisting of only the soluble ligand-binding domains and using these to measure affinity of binding, as has been done with S1,S2 domains from animal iGluRs (Kuusinen et al. 1995). In addition, the proposed signalling role of glutamate or glycine should be substantiated by high-resolution quantification of changes in apoplastic concentration (e.g. by fluorescence-based techniques; Acher et al. 2005; Fehr et al. 2005).

13.4.3 Knockout and Overexpression Phenotyping

As there are 20 *AtGLR*s, knockout of some members may result in subtle or no phenotypes owing to specificity of function or compensation by other members, making it necessary to reduce expression of several members simultaneously to readily identify phenotypes. This could be achieved by pyramiding knockouts or by employing an RNA interference strategy to silence several *At*GLRs simultaneously (Essah et al. 2005). Overexpression of *At*GLRs is also a desirable strategy, since it is possible

that excess of one subunit could disrupt the formation of normal heteromers and may have relatively widespread effects, which might be difficult to interpret but would give crude information about the processes in which *At*GLRs are involved. *At*GLRs have been suggested to have wide-ranging functions so phenotyping requires testing of plant responses to a range of different ligands (informed by Sect. 13.4.2), including glutamate, glycine, GABA, ABA, and other amino acids, as well as agonists and antagonists of animal iGluRs. Plant responses may involve germination, root growth, circadian-asssociated phenotypes such as aberrant hypocotyl angle or length, responses to light, nutrients and toxic ions, solute accumulation, and electrical and calcium signalling. Phenotyping needs to be informed to some extent by the reported physiological effects of amino acids on plant behaviour, but conversely physiological characterisation will depend partly on mutant characterisation to predict plant responses to ligands.

13.4.4 Heterologous Expression

Heterologous expression is desirable to demonstrate ion channel function of *At*GLRs. However expression in *Xenopus* oocytes has been uninformative (Sect. 13.3.3). Chimaeric iGluRs constructed by transplanting the pore domain of apparently non-functional iGluRs (such as KA-binding proteins and *C. elegans* iGluR) into rat iGluRs have been used to demonstrate functionality of the pore (Villmann et al. 1997; Strutz-Seebohm et al. 2003). Although not proof of function in vivo this approach could indicate whether the pore region is capable of ion conduction and its potential selectivity. Use of homologous or heterologous plant systems such as cultured mesophyll cells may result in correct processing. Alternatively the possible toxicity of some *At*GLRs in yeasts and *E. coli*, which suggests functional expression (Davenport 2002), could be used to advantage by inducible expression of toxic *At*GLRs, which would permit at least ion uptake studies. In some cases coexpression of subunits in heterologous systems may be required to produce functional ion channels. iGluRs function as heteromers and some subunits show no channel activity when expressed as homomers (Dingledine et al. 1999). The large size of the *At*GLR family and the evidence for simultaneous expression of many different *At*GLRs in single cells (Sect. 13.3.1) makes it difficult to predict likely heteromeric combinations, and it may be desirable to coexpress large pools of subunits simultaneously, or to use a yeast two-hybrid approach to identify interacting subunits.

13.4.5 NSCC Characterisation

The inadequacy of NSCC characterisation *in planta* and the uncertainty regarding the definitive identification of ions involved in (or the signalling components downstream of) the ligand gated *Em* depolarisation *in planta* make it difficult to match the characteristics of putative heteromer-dependent *At*GLR currents to native currents. The use of ion channel blockers and ion substitution in microelectrode impalements are producing some insights (Sivaguru et al. 2003; Qi et al. 2005) and greater resolution could be provided by further patch clamp experiments (Demidchik et al. 2004) where greater control of solutions could also reveal whether cytosolic components are involved. Selection of fluorescent protoplasts expressing marker:*At*GLR promoter fusions indicating expression of specific *At*GLRs within protoplasts may allow the tentative identification of currents with gene products (Kiegle et al. 2000). Alternatively, mRNA could be extracted from protoplasts after electrophysiological investigation and *At*GLR transcript abundance correlated with membrane conductance (Gehwolf et al. 2002).

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14 Similarities Between Endocannabinoid Signaling in Animal Systems and *N*-Acylethanolamine Metabolism in Plants

Elison B. Blancaflor, Kent D. Chapman

Abstract *N*-Acylethanolamines (NAEs) are minor lipid constituents of plant and animal cells, and their roles in mammalian physiology and neurobiology have been studied intensively for many years. However, corresponding studies on the function of NAEs in plants have appeared only recently. Within the last decade significant progress has been made in quantifying NAEs in plant tissues, characterizing their potential targets in plant cells and identifying the relevant enzyme involved in their degradation, but much remains to be determined regarding the role of these fatty acid amides in plant physiology. Although our understanding of the specific functions of NAE in plants is far from complete, recent advances in plant NAE biochemistry are pointing to intriguing similarities between animals and plants in the metabolism and perception of NAE. In this chapter we discuss NAEs as prospective signaling and regulatory molecules in plant cells. Advances in mammalian NAE research are presented when appropriate in order to draw parallels as well as to highlight differences between plant NAE metabolism and the endocannabinoid signaling system, the major pathway by which NAE exerts its physiological effects in animal cells.

14.1 Introduction and Overview of Mammalian Endocannabinoid Signaling

For many centuries derivatives of marijuana (*Cannabis sativa*) have been known to possess medicinal properties. Identification of 9-tetrahydrocannabinol (THC; Fig. 14.1a) as one of the active ingredients of marijuana led to the discovery of a group of G-protein coupled receptors that bind to THC. Two cannabinoid (CB) receptors, CB1 and CB2, have been cloned thus far, with CB1 being the most highly expressed neuromodulatory receptor in the brain. CB2 receptors, on the other hand, are absent in the brain but predominate in tissues of the immune system (Wilson and Nicoll 2002). Low levels of *N*-acylethanolamines (NAEs) were first identified in mammalian brain in 1965 (Bachur et al. 1965) but the function of this family of lipids remained unclear until *N*-arachidonylethanolamine (anandamide; NAE20:4; Fig. 14.1b) was discovered to be an endogenous ligand for CB1 receptors (Devane et al. 1992). This finding stimulated a flurry of research on the metabolism and biological activities of NAEs in several animal models, which eventually solidified the status of anandamide as an endogenous neurotransmitter (Self 1999). Anandamide as a ligand for

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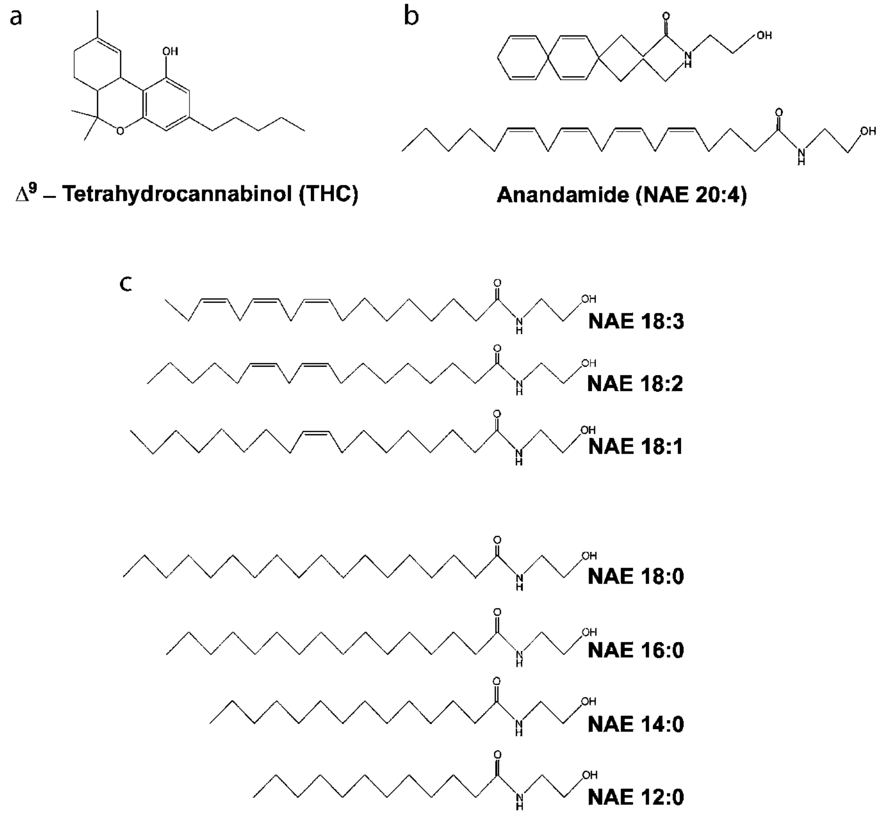

Fig. 14.1. Structures of selected cannabinoids. **a** Δ^9-Tetrahydrocannabinol, the major active ingredient in marijuana, is a strong ligand for cannabinoid 1 receptors in mammalian brains. **b** Anandamide is an *N*-acylethanolamine (*NAE*) with a 20-carbon acyl chain and four double bonds. It has a wide range of neuromodulatory and physiological functions in vertebrates, and is drawn in two different conformations. **c** NAE types identified and quantified in plants

CB receptors is somewhat surprising given the lack of obvious structural similarity with THC, but nearly all physiological effects elicited by marijuana have been attributed to anandamide and the metabolism of NAEs is now considered an integral part of the "endocannabinoid signaling system". The activity of anandamide at the CB1 receptor has been attributed by some (Reggio 2003) to its ability to adopt alternative conformations (Fig. 14.1b).

Although anandamide is the most studied NAE type in vertebrates, other endogenous NAEs such as *N*-oleoylethanolamine (NAE18:1) and *N*-palmitoylethanolamine (NAE16:0; Fig. 14.1c) elicit a number of biolog-

ical effects in animals, including satiety (LoVerme et al. 2005) and anti-inflammatory responses (Fowler 2003), respectively. Some of the cellular processes mediated by NAEs appear to be mediated by both receptor-dependent and receptor-independent pathways. For example, anandamide binding to CB1 receptors is known to stimulate $G_{i/o}$ proteins, which in turn influences the activity of a series of second messengers and proteins, including adenylate cyclase, voltage-gated calcium channels, K^+ channels, phosphatidyl 3-kinase and mitogen-activated protein (MAP) kinase (De Petrocellis et al. 2004; De Fonseca et al. 2005). On the other hand, experiments with mouse cell cultures showing that NAE induction of transcription and MAP kinase phosphorylation are not inhibited by $G_{i/o}$ protein blockers or CB1 receptor antagonists can be taken as evidence for receptor-independent signaling (Berdyshev et al. 2001).

The widespread occurrence of NAEs in various plant species (Chapman 2004), the recent cloning of a plant enzyme involved in the hydrolysis of NAE (Shrestra et al. 2003) and the identification of high-affinity NAE binding proteins in plant tissues whose binding appears to be influenced by CB receptor antagonists (Tripathy et al. 2003) point to intriguing parallels to mammalian endocannabinoid signaling. In this chapter, we review progress in plant NAE research focusing on metabolism and prospective functions while highlighting avenues for future work with the hope of generating excitement in this emerging field within plant physiology.

14.2 NAE Structure and Occurrence in Plants

NAEs are fatty acid derivatives with amide linkages to ethanolamine (Fig. 14.1). As such, they are electrically neutral, and as expected have limited solubility in aqueous solutions. Various NAE types have been identified in animals (Schmid et al. 1990), higher plants (Chapman 2004) and some microorganisms (Schmid et al. 1990). For the most part the acyl moieties of NAEs are a reflection of the fatty acid compositions of the parent organisms, although the precise profiles (and quantities) of NAEs in a given organism vary from tissue to tissue. Generally NAEs are present in amounts approximating nanograms per gram of fresh weight, but under certain pathological conditions, NAE levels accumulate to micrograms per gram of fresh weight (Schmid et al. 1990; Chapman 2004). This is particularly striking under conditions of ischemia in mammalian heart and brain, where NAEs accumulate in damaged tissues, but are barely detectable in nearby unaffected tissues (Schmid et al. 1990).

In plants, NAEs were first identified in processed, seed-derived products in the 1950s, but not until the 1990s did it become evident that

NAEs were endogenous metabolites in living plant cells. The NAE types identified in seeds were 12–18 carbons in length with zero to two double bonds, and the general abundance of these NAEs in desiccated seeds was on the order of micrograms per gram of fresh weight (Fig. 14.1c). Generally, *N*-linoleoylethanolamine (NAE18:2), NAE16:0, NAE18:1 and *N*-lauroylethanolamine (NAE12:0) were the four most abundant types of NAEs distributed in seeds, but their order of abundance varied in seeds of different plant species (Chapman et al. 1999). Moreover, improved quantitative methods have allowed for identification to be expanded to include *N*-linolenoylethanolamine (NAE18:3), which now can be classified as a common NAE present in most plant tissues. Total NAE content was found to vary over orders of magnitude in desiccated seeds from different legumes, but there was no obvious taxonomic relationship of NAE profiles (Venables et al. 2005). Instead NAE profiles seemed to reflect the general total fatty acid profiles in acyl lipids from the species of origin. Of particular significance is that the major NAE types that occur in plant tissues are identical to the major NAE types that occur in animal tissues, including NAE16:0, NAE18:1 and NAE18:2 – and the main differences occur amongst the minor NAE types such as NAE12:0 (reported only in plant systems) and NAE20:4 (reported only in animal systems).

14.3 NAE Metabolism in Plants

14.3.1 NAE Formation

In mammals, NAEs are derived from the hydrolysis of the membrane phospholipid, *N*-acylphosphatidylethanolamine (NAPE), by a Ca^{2+}-stimulated phospholipase D (PLD; Schmid et al. 1996, 2002; Fig. 14.2). A novel NAPE-selective PLD was cloned recently from mammals and the recombinant protein exhibited biochemical properties distinct from the well-characterized mammalian PLD1 and PLD2 proteins (Okamoto et al. 2004). The mammalian NAPE-PLD belongs to the zinc metallohydrolase family and exhibited activity only toward NAPE, and not toward the more abundant membrane phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Overexpression of this NAPE-PLD resulted in decreased NAPE levels and increased NAE levels (Okamoto et al. 2005), consistent with the notion that this enzyme hydrolyzes NAPE to form NAEs.

No obvious homologs of the mammalian NAPE-PLD are apparent in plants. However, radiolabeling experiments indicated that NAPE is the precursor for NAEs in plant cells and that the NAE types produced by plants are

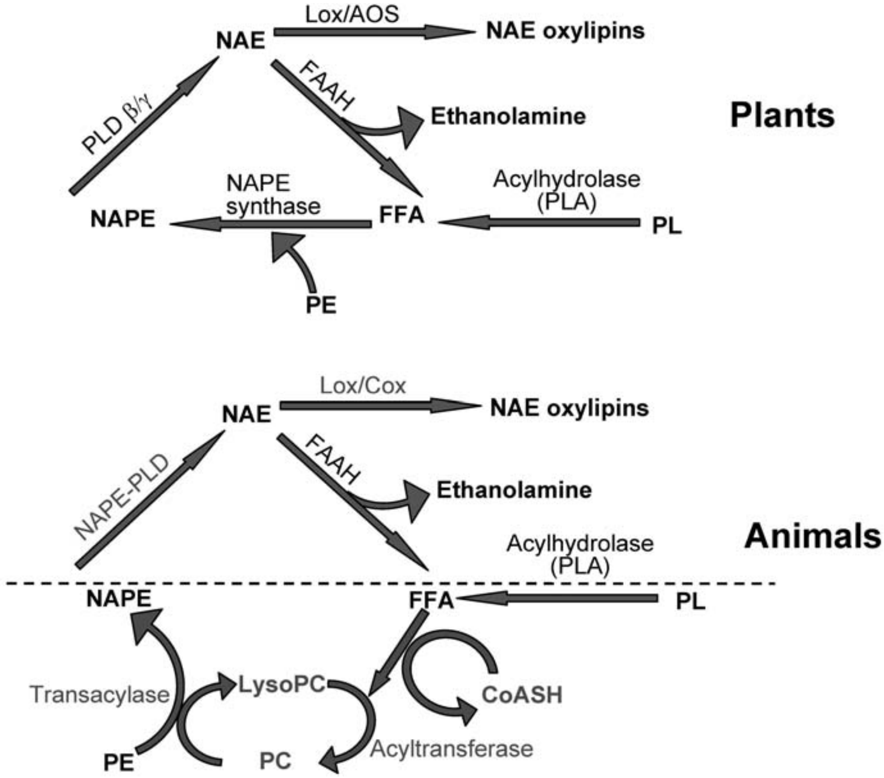

Fig. 14.2. Schematic comparison of NAE metabolism in plants and animals. NAEs are metabolized to NAE oxylipins (by lipoxygenase, *LOX*), or are hydrolyzed to free fatty acids (*FFAs*) and ethanolamine (by fatty acid amide hydrolase, *FAAH*) in both plant and animal systems. FFAs are incorporated into *N*-acylphosphatidylethanolamine (*NAPE*) directly by an NAPE synthase enzyme in plants, or by coordinated acyltransferase–transacylase reactions in animal systems. FFAs can be derived by FAAH-mediated NAE hydrolysis or from phospholipid acylhydrolases (*PLA*). NAPE is hydrolyzed by a phosphodiesterase activity to yield NAEs in both animal and plant systems. In plants, NAE formation is by an HKD/phosphatidyltransferase (PLDβ or PLDγ isoforms, where *PLD* is phospholipase D), whereas in animals NAE formation is by a specific NAPE-PLD zinc-metallohydrolase. *PC* phosphatidylcholine, *CoASH* coenzyme A, *PE* phosphatidylethanolamine, *AOS* allene oxide synthase, *COX* cylooxygenase. For clarity not all metabolites are shown

a reflection of the *N*-acyl composition of the NAPE precursor (Chapman 2000). Previous work demonstrated that two recombinant *Arabidopsis* PLD isoforms, PLDβ and PLDγ, were capable of hydrolyzing NAPE to NAE in vitro (Pappan et al. 1998). Five PLD isoforms (encoded by 12 genes) have been identified in *Arabidopsis* (Wang 2004). The most studied and most highly expressed PLD isoform in plants, PLDα, did not hydrolyze NAPE in

vitro (Pappan et al. 1998), but instead was inhibited by NAE in terms of its activity toward other phospholipid substrates. PLDδ did not form NAE from NAPE in vitro, and its activity toward other phospholipid substrates was not affected by NAEs (Chapman and McHugh, unpublished observations). PLDζ showed strict specificity for PC in vitro (Wang 2004), but NAPE was not tested as a potential substrate for this PLD family member. At this point the data suggest that PLDβ and/or PLDγ isoforms may catalyze the formation of NAEs in vivo, although this has not been demonstrated directly. Since there are two PLDβ genes and three PLDγ genes in *Arabidopsis* and these enzymes, unlike the mammalian NAPE-PLD, hydrolyze multiple phospholipid substrates, sorting out the precise PLD isoform(s) in plants responsible for NAE formation in vivo represents a considerable challenge.

A comparison of the mammalian machinery for NAE formation to that in plants reveals subtle but perhaps significant differences. While plants and animals both produce NAEs from NAPEs by phosphodiesterase activities (Fig. 14.2), the enzymes that catalyze this reaction operate by different mechanisms. The mammalian enzyme is a member of the zinc metallohydrolase family and hydrolyzes NAPE to NAE and phosphatidic acid (PA), but does not demonstrate transphosphatidylation activity that is characteristic of the PLD family of enzymes. The plant NAPE-PLDs belong to the HKD/phosphatidyltransferase family and display activity toward other phospholipid substrates in addition to NAPE (Wang 2002). These subtle differences may be found to have functional significance in the regulation of NAE formation in these two groups of multicellular eukaryotes.

14.3.2 NAE Hydrolysis

In mammalian systems, endocannabinoid signaling is believed to be terminated by the uptake of anandamide into the cell where it is hydrolyzed into arachidonic acid and ethanolamine (De Fonseca et al. 2005). The enzymatic degradation of NAEs in animal cells is facilitated by a single enzyme called fatty acid amide hydrolase (FAAH; McKinney and Cravatt 2005; Fig. 14.2). Cloning of rat FAAH in 1996 (Cravatt et al. 1996) eventually led to the identification of FAAH orthologs in other mammalian species. The mammalian FAAH enzymes are 579 amino acids in length and belong to a large group of proteins containing conserved amidase signature (AS) sequences. In contrast to other enzymes belonging to the AS family, FAAH is distinct in that it carries an N-terminal transmembrane domain that predicts it to localize to cellular membranes (Cravatt et al. 1996; McKinney and Cravatt 2005).

A breakthrough in plant NAE research was accomplished by the recent identification of a FAAH homolog (At5g64440) from the *Arabidopsis* genome based upon the occurrence of the AS domain and conservation of several key catalytic residues (Shrestha et al. 2003). The *Arabidopsis* FAAH complementary DNA (cDNA) was predicted to encode a protein of 607 amino acids and the AS domain was nearly 60% identical to that of the mammalian FAAH. Moreover, like mammalian FAAHs, the *Arabidopsis* FAAH protein contained a putative transmembrane domain near the N-terminus and shared three domains of unknown function near the C-terminus (Shrestha et al 2003). The recombinant *Arabidopsis* FAAH was shown by biochemical analysis to hydrolyze a variety of plant NAEs and, interestingly, had a higher affinity for anandamide. Furthermore, *Arabidopsis* FAAH activity was strongly inhibited by methylarchidonyl fluorophosphonate, an active-site-directed inhibitor of mammalian FAAH (Shrestra et al. 2003). Thus, despite the low primary sequence similarity between mammalian and *Arabidopsis* FAAH (just 18.5% identity over their entire lengths), molecular and biochemical evidence indicate that the *Arabidopsis* At5g64440 gene encodes a functional NAE amidohydrolase. Hence, the machinery for degradation of NAEs appears to be functionally conserved between plants and animals (Fig. 14.2).

The cloning of mammalian FAAH paved the way for the generation of animal models to characterize the role of this enzyme in endocannabinoid signaling. As predicted, FAAH knockout mice exhibited a tenfold elevation of anandamide and other NAEs when compared with wild-type mice. In addition to exhibiting increased sensitivity to exogenously applied anandamide, FAAH knockout mice displayed a variety of physiological and behavioral abnormalities consistent with altered endocannabinoid signaling, such as hypothermia and reduced response to pain (Cravatt et al. 2001). Similar to the scenario in FAAH knockout mice, *Arabidopsis* seedlings downregulating or overexpressing FAAH are altered in their sensitivity to exogenously applied NAE. Preliminary results with FAAH transgenics are beginning to indicate that the formation and degradation of NAE may be important in mediating plant responses to the environment (Wang, Blancaflor and Chapman, unpublished data).

14.3.3 NAE Oxidation

NAE action in plants could be accomplished via the formation of oxylipins, which are cyclic oxidation products derived from the catabolism of free fatty acids (FFAs). In mammalian systems, oxidation products of polyunsaturated NAEs, including eicosanoid ethanolamides, prostaglandins and

leukotrienes, comprise an important class of signaling molecules that participate in diverse physiological processes (De Petrocellis et al. 2004). As in the case of animals, polyunsaturated NAEs found in plants such as NAE18:2 and NAE18:3 can serve as substrates for enzymatic oxidation by the lipoxygenase (LOX) pathway to produce novel plant oxylipins (van der Stelt et al. 2000; Fig. 14.2), raising the possibility that NAE oxylipins may mediate certain physiological processes in plants. In fact, jasmonic acid and its methyl esters represent a class of plant oxylipins that function as signaling compounds during different stages of plant development (Turner et al. 2002). Also, 12-oxo-13-hydroxy octadecenoylethanolamide is formed during cottonseed imbibition but the physiological significance of these NAE-derived metabolites remains unclear (Shrestha et al. 2002). Additional work is needed to fully understand the significance of NAE oxylipin formation during seed germination and response to pathogens.

14.3.4 NAPE Formation

NAPE is the membrane phosph olipid precursor for NAE, and the types of NAEs that are formed are almost always a reflection of the acyl groups present in the NAPE precursor pool (Schmid et al. 1996). In other words there is little, if any, selectivity for NAPE molecular species by the NAPE-PLDs (Okamoto et al. 2004, 2005). This suggests that the synthesis of NAPE precursors may play an important part in the endocannabinoid signaling pathway by determining the profile of available NAEs.

The synthesis of NAPE in animal systems proceeds by an energy-independent transacylation reaction whereby the *sn*-1-*O*-acyl moiety of PC is transferred to the N-position of PE without a FFA intermediate (Schmid and Berdyshev 2002). This transacylase activity has been characterized in vitro in several animal systems, but has not yet been cloned from any organism. Because arachidonic acid is seldom found at the *sn*-1-position of mammalian PC, some controversy has developed over this proposed mechanism in terms of its ability to account for the accumulation of anandamide. It is generally believed that acyl groups (including arachidonic acid) are shuttled into the *sn*-1 position of lysoPC from a fatty acid pool generated by phospholipase A activity on membrane phospholipids (Fig. 14.2). Hence, this proposed pathway involves the incorporation of fatty acids into the N-position of NAPE by the coordinate action of acyltransferase and transcylase activities. By contrast, plants synthesize NAPE directly from FFAs, by a membrane-bound enzyme designated NAPE synthase (Chapman 2000). Notably, acyl moieties from acyl coenzyme A or PC were 100–1,000 times less efficiently incorporated in NAPE than unesterified FFAs (Chapman

and Moore 1993). The NAPE synthase enzyme has been purified to homogeneity from cottonseed microsomal membranes, but no cDNA sequences have been identified yet that encode this enzyme. Because this enzyme utilizes FFAs and PE, two membrane bilayer-destabilizing lipids, as substrates, and synthesizes NAPE, a bilayer-stabilizing lipid (Schmid et al. 1990), this might be a mechanism for scavenging FFAs and protecting membrane integrity during plant cell stress. In fact work in potato cells suggests that this mechanism may operate in vivo wherein FFAs accumulate in response to hypoxic stress, followed by a rapid elevation of NAPE (Rawyler and Braendle 2001).

Plants and animals ultimately synthesize NAPE using the same metabolites (FFA and PE), but have developed different enzymatic machinery to accomplish this synthesis. These metabolic differences might relate to different mechanisms of regulation of overall NAE metabolism in plants and animals. Reconciliation of these proposed pathways and a better understanding of the role of NAPE biosynthesis in NAE signaling will be greatly enhanced by the molecular identification of DNA sequences encoding the enzymes involved in this pathway.

14.4 Prospective Functions of NAE in Plants

14.4.1 NAEs in Plant Defense Responses

As noted already, the accumulation of NAEs under neurodegenerative conditions led to the proposal that NAEs might have neuroprotective roles in animals (Fowler 2003). Evidence supporting the notion that plants and animals might share common NAE signaling pathways comes from the observation that plant cells also accumulate NAEs in response to stress (Chapman et al. 1998). For instance, the fungal elicitor xylanase stimulated tobacco suspension cells to release NAEs into the culture medium. However, in contrast to mammalian cells, which released mostly long-chain, saturated and unsaturated acyl chain NAEs (NAE16:0 and NAE20:4; Berger et al. 2004), the NAEs that accumulated following fungal elicitation were of the shorter acyl chain types (NAE12:0 and *N*-myristoyethanolamine, NAE14:0; Chapman et al. 1998). This could be reflective of differences in the physiological effects and/or targets of these NAE types in plants and animals. In mammalian systems, the action of one NAE type modulates the activity of another NAE species by prolonging its signaling activity (Fowler 2003). This "entourage" effect is best illustrated with NAE16:0 and

NAE20:4. Although NAE16:0 does not bind to CB1 receptors, it is capable of downregulating FAAH expression, leading to the enhancement of anandamide effects in the cell (De Petrocellis et al. 2002). It will be interesting to see whether a similar scenario occurs between the different NAE types in plants.

Tobacco cell cultures exhibit a short-term alkalinization of the culture medium upon exposure to xylanase (Felix et al. 1993). When added without elicitors, NAE14:0 did not affect the alkalinization response, but when added together with xylanase, the elicitor-induced alkalinization response was inhibited. Most plant NAE types, including the mammalian NAE anandamide, showed this inhibitory effect toward xylanase-induced alkalinization (Tripathy et al. 1999). Moreover, NAE14:0 inhibited the alkalinization response induced by other pathogen elicitors and the effect of added NAE14:0 was dependent on concentration as well as the timing of addition (Tripathy et al. 1999).

There is also evidence that NAEs participate in gene expression changes in plants responding to pathogens. The induction of phenylalanine ammonia lyase (PAL) gene expression, a critical enzyme in the phenylpropanoid pathway, typically accompanies pathogen attack (Dixon et al. 2002). These changes in PAL transcript abundance are likely related to the cell's ability to establish a coordinated defense response to ward off the invading pathogen. Interestingly, PAL2 gene expression was induced by NAE14:0 in a manner that mirrored the induction by elicitors. More importantly, NAE14:0 accumulated 10–50-fold in elicitor-treated tobacco leaves and these concentrations were capable of activating PAL2 gene expression. On the basis of these observations it is possible that NAE formation following elicitor treatment is part of the signal transduction machinery that leads to plant defense responses.

As discussed earlier, NAEs in vertebrates are perceived by transmembrane CB receptors at the cell surface (Wilson and Nicoll 2002). A similar mechanism for NAE perception might exist in plants to facilitate signaling during pathogen elicitor perception. Evidence in support of this assumption comes from the recent identification of a high-affinity NAE binding protein in cell suspensions and microsomal membranes from a variety of plant sources (Tripathy et al. 2003). Interestingly, CB receptor antagonists were able to reduce NAE14:0-specific binding when included in the assays and these antagonists diminished the NAE14:0-induced PAL2 expression in leaves of tobacco plants as well as the NAE inhibition of the short-term elicitor-induced alkalinization response (Tripathy et al. 2003). Taken together these results support the concept of a CB receptor-like NAE binding protein participating in defense signal transduction. The successful isolation of a functional protein and the generation of knockouts/overexpressors for this binding protein will represent an invaluable

step toward determining whether NAE receptors are responsible for mediating NAE effects during plant defense signaling.

14.4.2 NAE in Seed Germination and Seedling Growth

NAEs and their precursor NAPEs are actively metabolized during seed germination (Sandoval et al. 1995; Chapman et al. 1999; Shrestha et al. 2002), raising the possibility that the rapid metabolism of these fatty acid ethanolamides during seed imbibition is necessary for the establishment of normal seedling growth. Indeed, one of the more dramatic biological effects of NAEs in plants is the recent observation that exogenous NAE12:0 impairs normal seedling development in *Arabidopsis*. These defects in seedling development included a reduction in primary root elongation, inhibition of root hair initiation and an alteration of cell division patterns in the root meristem. The disruption of normal root development was reflected at the cellular level as defects in endomembrane dynamics, and microtubule and actin organization (Blancaflor et al. 2003; Motes et al. 2005). Interestingly, 1-butanol, a nonselective inhibitor of PLD because it blocks the transphosphatidylation reaction catalyzed by all PLDs, is capable of inducing similar defects in root development as NAE12:0 (Gardiner et al. 2003; Motes et al. 2005). The previously mentioned studies suggest that some of the physiological effects of NAE on seedling development could be related to impaired PLD function. Indeed, downregulating PLDζ through interference RNA induces abnormalities in root hair development (Ohashi et al. 2003) and knockouts to a gene involved in PC synthesis, an intermediate lipid in the synthesis of PA by PLD, leads to defects in root development (Cruz-Ramirez et al. 2004). The effect of 1-butanol on the organization of the microtubule cytoskeleton has been suggested to be the result of a two-step transphosphatidylation reaction catalyzed by PLD (Dhonukshe et al. 2003).

Perhaps a more convincing argument supporting PLD as a target of NAE during seedling development is a report showing that NAEs are potent, noncompetitive inhibitors of PLDα activity in vitro. Consistent with these results is the observation that low concentrations of NAE inhibit abscisic acid induced stomatal closure in leaf epidermal peels (Austin-Brown and Chapman 2002), a process that is known to be mediated by PLDα (Zhang et al. 2004). A closer analysis of the effects of 1-butanol and NAE on specific cellular processes as well as studies of single or multiple PLD knockouts (Wang 2002) particularly with regard to their responses to NAE should provide clues as to how NAE interacts with PLD in modulating seed germination.

14.5 Conclusions and Future Prospects

Since the discovery of anandamide as an endogenous neurotransmitter, there has been unprecedented progress in our understanding of NAE signaling in animals. As a result of these discoveries, new drugs have been designed that could potentially be used to treat medical conditions resulting from compromised endocannabinoid signaling (De Fonseca et al. 2005). Although plant NAE research is still in its infancy, it appears that plants share many important features of animal endocannabinoid signaling (Fig. 14.2). For example, the accumulation of NAEs during pathogen elicitation and anoxic stress parallels the elevation of NAEs seen in mammalian cells undergoing neurological degeneration. Also there is evidence that plants, like animals, metabolize NAEs via amidohydrolase and LOX pathways. Finally, perception of NAE in plants may be facilitated by transmembrane proteins that could function in a manner analogous to the CB receptors in mammals. We anticipate that studies in this historically underpopulated field would be bolstered by new approaches such as lipid metabolic profiling (Saghatelian et al. 2004) and the more traditional forward/reverse genetic techniques to alter specific steps in NAE metabolism and perception. Moreover, the combination of sensitive quantitative procedures for endogenous NAE metabolites along with the identification of conditions that influence NAE accumulation should remain an important area of focus in the future.

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15 Regulation of Plant Growth and Development by Extracellular Nucleotides

Stanley J. Roux, Charlotte Song, Collene Jeter

Abstract The traditionally emphasized roles of ATP and other nucleotides are in energy metabolism, and they all happen within the borders of the plasma membrane of cells. However, recent findings, first in animals and then in plants, indicate that ATP and ADP can have significant roles outside the plasma membrane as agonists that do not have to be hydrolyzed to induce diverse signaling responses in cells. This chapter reviews results obtained mainly in *Arabidopsis* that point to a likely role of extracellular nucleotides as regulators of plant growth and development. Concentrations of ATP in the 40–60-μM range are released from cells into intercellular spaces of plant tissues by wounds, and lower levels of applied ATP, but not AMP or phosphate, can induce increases in the concentration of cytoplasmic calcium ions and such calcium-dependent downstream responses as increased superoxide production and increased levels of messenger RNAs associated with wound responses such as lipoxygenase and 1-aminocyclopropane-1-carboxylic acid synthase. Inhibitors of calcium signaling (calcium chelators, La^{3+}, Gd^{3+}, calmodulin antagonists) and inhibitors of animal purinoceptors suppress the inductive effects of ATP in plants. Because growth zones of plants are regions of high secretory activity and because secretory vesicles contain millimolar levels of ATP that are delivered to the plant extracellular matrix when they fuse with the plasma membrane, extracellular ATP could also play a signaling role during growth of unwounded tissues, and published evidence for this is reviewed and discussed here. Although extracellular nucleotides are well-established agonists in animal cells, the evidence for their role in plants is thus far only indirect. The discovery and characterization of purinoceptors was the breakthrough that confirmed extracellular nucleotides were signaling agents in animals, and a similar identification of ATP-/ADP-responsive receptors in plants will be required to confirm that these agents are extracellular regulators of plant metabolism.

15.1 Introduction

Although most biologists think of ATP as the principal energy currency of the cell, functioning entirely inside of cells, there is a significant literature documenting an extracellular function of ATP as an agonist that does not have to be hydrolyzed to activate responses in cells (Burnstock and Knight 2004). This literature originally was restricted to studies of animal cells, where adenine nucleoside triphosphate and diphosphate mediate a wide variety of biological processes in the extracellular matrix (ECM) at specialized receptors known as P2 purinoceptors. The main types of these multigene family receptors in animals are P2X, 2-pass transmembrane (TM) subunits which oligomerize to form ligand-gated ion channels, and P2Y,

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7-pass TM heterotrimeric G-protein linked receptors. The binding of ATP or ADP (among other NTPs and NDPs) activates these receptors, initiating secondary messenger systems and downstream signaling cascades, thereby affecting changes in gene expression and culminating in the induction of cell-type specific responses.

The first recognized physiological activity of these signaling agents was that of a co-neurotransmitter, and so this type of signaling was originally known as purinergic transmission (Burnstock 1972). Derivatives of ATP, including ADP and adenosine, were also shown to have biological effects. For example, adenosine functions as a negative regulator of neurotransmitter release at specialized P1 purinceptors, functioning together with ATP to modulate smooth muscle contraction; and ADP signals blood platelet aggregation during thrombosis (reviewed in Burnstock 1996; Ralevic and Burnstock 1998).

Extracellular ATP (eATP) has been reported to have numerous effects on the physiology of plants, too, altering both developmental programs and responses to environmental stimuli. Early studies showed that exogenous application of ATP could induce the closure of the Venus's-flytrap (Jaffe 1973), affect cytoplasmic streaming in *Chara* cells (Williamson 1975), modulate stomatal aperture in *Commelina communis* (Nejidat et al. 1983), and stimulate pollen tube generative nuclear divisions in *Lilium lingiflorum* (Kamizyo and Tanaka 1982). However, most of these reports assumed that the applied ATP was somehow, directly or indirectly, altering the energy charge of the cell, and thus was still playing its standard role of driving energy-dependent reactions.

More recent results have revealed that the hydrolysis of eATP is not required to induce responses in plant cells (Demidchik et al. 2003; Jeter et al. 2004). This has led plant scientists to recognize the potential role of eATP as an agonist, exerting its effects through interaction with cell surface receptors, similar to what happens in animal cells. The purpose of this chapter is to review this more recent literature and discuss potential mechanisms by which extracellular nucleotides could alter plant growth and development as cell–cell signaling molecules.

15.2 Rapid Responses of Plants to Applied Nucleotides

15.2.1 Induced Changes in the Concentration of Cytoplasmic Calcium Ions

In animal cells, activation of purinoceptors by extracellular nucleotides rapidly leads to changes in membrane potential and increases in the con-

centration of cytoplasmic calcium ions ($[Ca^{2+}]_{cyt}$). Not surprisingly, then, scientists interested in testing the signaling roles of eATP and extracellular ADP (eADP) in plants assayed the effects of these nucleotides on membrane transport properties.

The first report of the rapid induction of membrane potential changes by extracellular nucleotides was that of Lew and Dearnaley (2000), who demonstrated that both eADP and eATP could induce large membrane depolarization changes in root hairs of *Arabidopsis thaliana* within seconds after the application. Phosphate application had no effect, negating the explanation that the depolarization was the result of phosphate released by hydrolysis of the applied nucleotides. Applied ATP, GTP, and ADP all induced large depolarizations, but AMP, TTP, and CTP did not. Dose-response tests revealed that half-maximal depolarization happened at 0.4 mM for ATP, but at only 10 μM for ADP, indicating that eADP was the more effective inducer of this response. Given that in animals nucleotide binding induces increased $[Ca^{2+}]_{cyt}$, whether the receptor is either a P2X or a P2Y type, the authors tested for a change in $[Ca^{2+}]_{cyt}$. Using dextran-conjugated calcium green as the reporter of $\Delta[Ca^{2+}]_{cyt}$, they reported no change in $[Ca^{2+}]_{cyt}$ induced by either eATP or eADP. An increase in $[Ca^{2+}]_{cyt}$ would be expected to decrease cytoplasmic streaming in the root hairs, but applied nucleotides had no effect on this, either. Interestingly, eADP, but not eATP, induced a slight (22–38%) increase in root hair growth.

Even 10 μM ADP is a much higher concentration than would be expected in the ECM of any plant or animal cell, so Lew and Dearnaley (2000) speculated that the most likely naturally occurring situation that would expose root hairs to high concentrations of eATP would be root wounding, which would release cytoplasmic ATP to the outside of the cell. Several authors have measured cytoplasmic ATP concentrations at between 1 and 2 mM (Gout et al. 1992). Although ATP released from root cells by wounding would be rapidly hydrolyzed by wall-localized apyrases and phosphatases, it could be expected to reach and remain above 10 μM long enough to induce membrane depolarization changes.

If extracellular nucleotides were activating receptors in root hair cells, their failure to induce changes in the $[Ca^{2+}]_{cyt}$ of these cells suggested that the signaling pathways they induced were different from those induced by purinoceptors in animals. Alternatively, it was possible that the experimental setup used by Lew and Dearnaley was not sensitive enough to detect the changes in $[Ca^{2+}]_{cyt}$ induced by eATP and eADP. Demidchik et al. (2003) tested this possibility by using a very different methodology to assess the effects of extracellular nucleotides on $[Ca^{2+}]_{cyt}$. They used transgenic *Arabidopsis* plants constitutively expressing apoaequorin (Knight et al. 1996). When these plants take up the luminophore coelenterazine, the apoaequorin they are expressing is converted to the bioluminescent calcium

sensor aequorin, which can then sensitively report changes in $[Ca^{2+}]_{cyt}$ by giving off light.

Demidchik et al. (2003) applied nucleotides to excised roots bathed in a buffered solution containing 10 mM $CaCl_2$ and found that as little as 300 nM ATP could induce a twofold increase in $[Ca^{2+}]_{cyt}$ in less than 10 s. The nonhydrolyzable 2-meATP was almost as effective as ATP, indicating that agonist hydrolysis was not required for the response (Fig. 15.1). Neither AMP nor phosphate induced any significant change in $[Ca^{2+}]_{cyt}$, and the pyrimidine UTP was ineffective below 100 μM, demonstrating the specificity of the nucleotide action. The P2-receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and the calcium channel blocker gadolinium, both of which inhibit eATP action at some P2 receptors in animal cells, completely suppressed the response of roots to eATP. Taken together, the dose responsiveness, substrate specificity and pharmacological profile suggested that the response was mediated by a distinct cell surface receptor, possibly an ion channel. The magnitude of the increase in $[Ca^{2+}]_{cyt}$, from approximately 100 nM to over 400 nM after treatment with 1 μM ATP, was certainly sufficient to turn on calcium-dependent signaling pathways in plants.

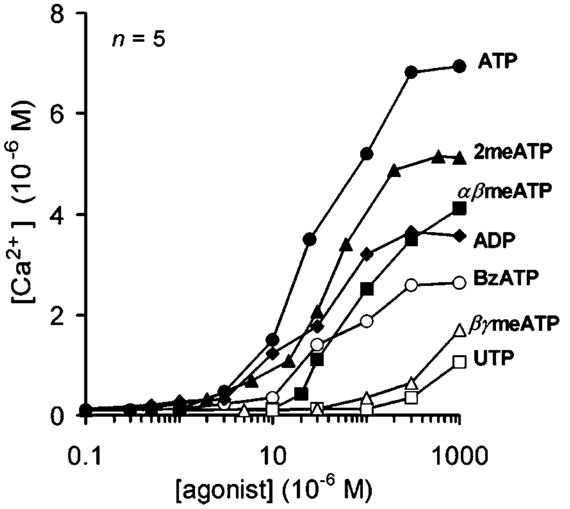

Fig. 15.1. Externally applied purines and UTP induce an increase in the concentration of cytoplasmic calcium ions ($[Ca^{2+}]_{cyt}$) in *Arabidopsis* roots. Shown are the mean dose-response curves for effects of different agonists on $[Ca^{2+}]_{cyt}$, as estimated by aequorin luminescence. ATP analogs tested included BzATP, an analog with a modified Rib moiety; *αβ*meATP, which has a methylene insert between the first and second phosphates; and *βγ*meATP, which has a methylene insert between the second and third phosphate. (Taken from Demidchik et al. 2003, with permission)

Differences between the results of Lew and Dearnaley (2000) and Demidchik et al. (2003) can be attributed mainly to the different methodologies used (e.g., intact vs. excised roots; root hair response vs. whole root response; calcium green vs. aequorin reporter), but both results argued strongly for the conclusion that ATP is a signaling agent in plants, just as it is in animals. Both results, however, begged the question: Do these nucleotide-induced changes lead to downstream responses that are known to be regulated by $\Delta[Ca^{2+}]_{cyt}$?

A clear answer to this question came from the results of Jeter et al. (2004). These authors, using the same type of aequorin-expressing *Arabidopsis* plants employed by Demidchik et al. (2003), found that applied nucleotides could induce significant increases in $[Ca^{2+}]_{cyt}$ in intact *Arabidopsis* seedlings, with most of the luminescent signal coming from the aerial parts of the plant. They used similar controls as Demidchik et al. (2003), including all of the naturally occurring ATP derivatives such as AMP and phosphate, but using different poorly hydrolyzable nucleotide P2-receptor agonists (ATPγS, ADPβS, and AMPS). They observed similar results, demonstrating other plant tissues besides the root can also respond to exogenously applied nucleotide derivatives, and confirming the specificity of the nucleotide effects. Further studies with calcium flux inhibitors, especially the use of the calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA), strongly argue for an influx of calcium from the ECM, and, together with previous reports, suggest the presence of a plasma membrane-localized ion channel mediating the increased $[Ca^{2+}]_{cyt}$. The magnitude of the $\Delta[Ca^{2+}]_{cyt}$ induced by nucleotides in intact seedlings was lower than that observed by Demidchik et al. (2003) in excised roots (Jeter et al. 2004). This and the apparent higher threshold for induction observed in the seedlings may have been due to intrinsic differences in the responsiveness of the two tissues, or to differences in the protocols employed (e.g., excised vs. intact tissue; 10 mM Ca^{2+} in measuring medium for roots vs. less than 100 μM Ca^{2+} in measuring medium for seedlings).

Jeter et al. (2004) also documented that the eATP treatments induced downstream gene expression changes known to influence hormone and stress responses, thus linking the initial $[Ca^{2+}]_{cyt}$ changes to later genetic changes that could mediate the growth and development of responding plants. The application of 500 μM ATP or ADP, but not that of AMP or buffer, induced an increased abundance of messenger RNAs encoding various mitogen-activated protein kinases (ATMEKK1, ATMPK3, ATPK19), and the ethylene-related ERF2, ERF3, ERF4, and ACS6 genes. These gene expression changes were partially blocked in cells pretreated with Gd^{3+} or a calcium chelator, revealing their dependence on an increase in $[Ca^{2+}]_{cyt}$. The same genes had been shown to be up-regulated by touch and osmotic stresses

(Arteca and Arteca 1999; Reymond et al. 2000), mechanical stimuli known to induce animal cells to release ATP into the ECM (Sauer et al. 2000), so the authors tested whether these mechanical stresses could induce the same tissues that respond to externally applied nucleotides to release ATP.

As assayed by the sensitive luciferin–luciferase method, stress stimuli do indeed induce measurable ATP release from young seedlings (Jeter et al. 2004; Fig. 15.2). The touch stimulus was applied by gently shaking the seedlings, and the hypertonic stress was applied by briefly submerging the seedlings in 300 mM NaCl. Seedlings recovered from the stresses applied and appeared normal within 24 h, indicating that it was unlikely the released ATP came from damaged cells.

Pathogen attack is another form of stress, and plants typically respond to this stress by the release of oligogalacturonides (OGA) in their ECM. This OGA serves as a signaling molecule to induce defense responses through a transduction pathway that includes increase in $[Ca^{2+}]_{cyt}$ as an early step. Since ATP release from damaged cells would be another likely event induced by pathogen attack, could ATP and OGA function together to elicit wound responses?

Jeter et al. (2004) investigated this point and found that OGA and ATPγS mutually enhanced each other's effects on the $\Delta[Ca^{2+}]_{cyt}$. ADPβS with OGA similarly increased $[Ca^{2+}]_{cyt}$ together, whereas AMPS had a significant inhibitory effect. These results suggested that nucleotide derivatives could function together with OGA to modulate plant responses to wounding or herbivory, but they raise the question of the relationship between ATP and OGA signaling pathways.

Cessna and Low (2001) concluded that OGA induces increased $[Ca^{2+}]_{cyt}$ by Ca^{2+} release from internal stores, whereas the results of both Demid-

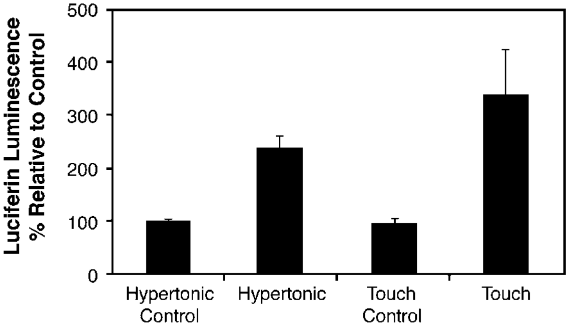

Fig. 15.2. Various stress stimuli induce ATP release from *Arabidopsis* seedlings. The relative ATP level in the medium surrounding the seedlings was measured using the luciferin–luciferase assay (Taken from Jeter et al. 2004, with permission)

chik et al. (2003) and Jeter et al. (2004) showed that ATP induces increased $[Ca^{2+}]_{cyt}$ primarily by promoting Ca^{2+} uptake from the ECM. Thus, pathogen attack appears to lead to the release of at least two different signaling agents, ATP and OGA, that act by different pathways to reinforce each other's stimulation of an early defense response, increased $[Ca^{2+}]_{cyt}$, that is critical for downstream defense activities of the plant.

15.2.2 Induced Changes in Superoxide Production

This theme of ATP involvement in defense responses is supported further by the pioneering work of Song and collaborators (Song 2004; Song et al. 2004). They investigated whether the level of ATP that accumulates in the apoplastic space of a wound site is sufficient to induce superoxide production and downstream wound signaling responses. They used micropipettes to collect multiple samples (several hundred femtoliters each) of fluid accumulating at wound sites of *Arabidopsis* leaves and measured the concentration of ATP in these samples using the luciferin–luciferase method. They found that the concentration was consistently in the 30–50 μM range.

Song (2004) also found that delivery of ATP samples as low as 500 nM into the intercellular spaces of *Arabidopsis* leaves was sufficient to rapidly induce significant superoxide production in them, as measured by the colorimetric method of Jabs et al. (1996), which uses nitroblue tetrazolium as the staining agent. Delivery of equivalent concentrations of phosphate buffer or of AMP into the leaves had no significant effect on this response. That the response required the participation of NADPH oxidase, a key enzyme that catalyzes superoxide production in plants (Mahalingam and Federoff 2003) and in animals (Vignais 2002), was demonstrated by the authors' observation that mutants disrupted in two genes that encode subunits of an NADPH oxidase homolog also do not accumulate significant superoxide in response to eATP (Song et al. 2004).

Various inhibitor treatments provided insight into the signaling pathway leading from ATP to superoxide production. Inhibitors of receptors that initiate eATP responses in animals, such as PPADS, were able to block the response. Cation channel blockers, calcium chelators, and calmodulin antagonists also blocked this ATP response, implicating increases in $[Ca^{2+}]_{cyt}$ and the activation of calmodulin as intermediate signaling steps (Fig. 15.3). Perhaps most importantly, pretreatment of leaves with relatively low concentrations of potato apyrase (Sigma), an enzyme that efficiently hydrolyzes ATP, significantly reduced the level of superoxide induced by wounding, arguing that it is likely ATP itself, and not a breakdown product, that elicits the response (Wang and Roux, unpublished).

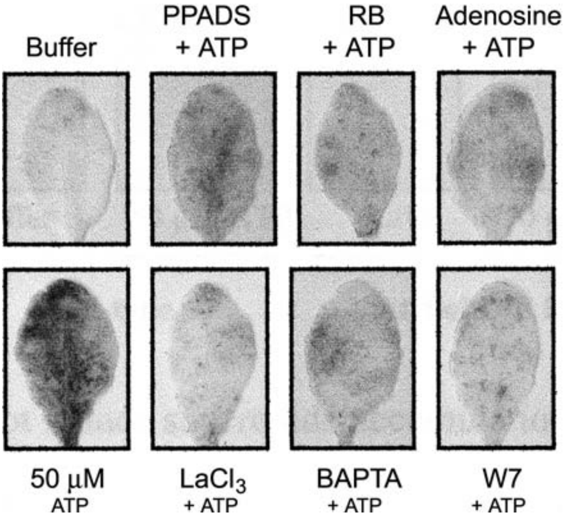

Fig. 15.3. Inhibitors of animal P2 receptors, Ca^{2+} signaling, and adenosine reduce the superoxide accumulation induced by ATP. Shown are images of representative *Arabidopsis* leaves stained for superoxide production (Jabs et al. 1996) after they had been treated with buffer, ATP, or ATP plus inhibitor

Genes that are induced by various stresses, including genes involved in the biosynthesis of jasmonates and ethylene, *LOX2* and *ACS6*, respectively, were up-regulated by eATP at the same micromolar concentrations that induced superoxide production, further supporting a role for eATP as a signal. Message abundance for *PAL1*, which is a well-studied stress-induced and reactive oxygen species induced gene, was also increased by eATP, and this effect was blocked by P2-receptor antagonists (Song 2004).

Taken together these results strongly suggest that the release of ATP at wound sites can serve as an early signal to induce superoxide production and downstream gene expression changes typically induced by the wound stimulus. More rigorous support for this hypothesis would require testing whether the knockout of genes encoding enzymes that could regulate the eATP concentration at wound sites, such as ectoapyrase enzymes (Thomas et al. 2000), alter the occurrence and intensity of signaling steps normally induced by the wounding stimulus.

15.3 Slower Growth Response Changes Induced by eATP

Given that superoxide production can have growth regulatory effects that can be both promotive and inhibitory of growth (Liskay et al. 2004; Mittler

et al. 2004), this leads to the question of whether eATP, which can induce superoxide production when applied to unwounded leaves, has any growth effects. Tang and colleagues (Tang et al. 2003; Tang 2004) provided an initial answer to this question. They demonstrated that relatively high concentrations of applied ATP and ADP (3 mM) and lower concentrations of the relatively nonhydrolyzable nucleotides ATPγS and ADPβS (0.3 mM) could inhibit the straight growth of roots, and that concentrations of these nucleotides about 3 times lower could inhibit gravitropic growth of roots without significantly inhibiting their straight growth.

Because of the strong relationship of auxin transport to growth, Tang et al. (2003) tested whether the growth inhibition induced by applied ATP could be mediated by the eATP effects on auxin transport. Their results indicated that both in maize and in *Arabidopsis* roots the same concentrations of ATP that inhibited gravitropic growth also inhibited auxin transport.

To investigate by what mechanism ATP was having its effects on growth, Tang et al. (2003) carried out a variety of controls to render unlikely some trivial explanations, such as that the effects were due to ATP-induced pH changes or chelation of divalent cations or to phosphate released from the hydrolysis of the applied nucleotides. They proposed two possible mechanisms of ATP action. One, based on the results of Thomas et al. (2000), was that eATP could reduce the steepness of the ATP gradient across the plasma membrane and thus inhibit the transport effectiveness of a multidrug resistance transporter that has been implicated in auxin transport (Noh et al. 2001). The other was that the applied ATP could be acting through the more traditional mechanism of activating a P2 purinoceptor.

Regarding the receptor hypothesis, none have been identified so far in any plant. If one were identified as mediating the growth effects reported by Tang et al. (2003), either it would have to be far less sensitive than the mammalian ones, or only a small fraction of the applied ATP and ADP would be reaching the receptor site, with the rest being rapidly hydrolyzed or otherwise altered. In the animal literature P2X purinoceptors were originally thought to be quite insensitive, responding only to millimolar levels of ATP, but now it appears that P2X receptors can respond to nanomolar levels of ATP, but these same levels desensitize the receptors so that they subsequently will respond only to much higher (millimolar) doses (Rettinger and Schmalzing 2004). The same desensitization phenomenon could significantly raise the threshold of plant responsiveness to external nucleotides.

Schopfer (2001) has presented data favoring a promotive role for superoxide in plant growth. Can the low levels of eATP that promote superoxide production in *Arabidopsis* leaves promote growth of *Arabidopsis*? Tang (2004) showed that concentrations of ATP in the range 100–200 μM could significantly promote hypocotyl growth in etiolated seedlings of *Arabidop-*

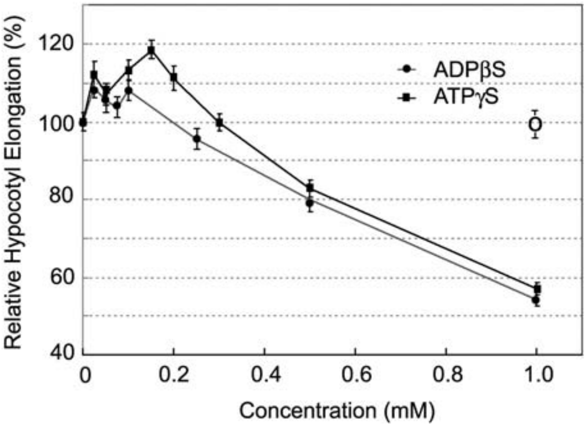

Fig. 15.4. Effects of applied of ATPγS and ADPβS on the elongation of hypocotyls of etiolated *Arabidopsis* seedlings. The *open circle* at 1.0 mM indicates that at this concentration AMP had no effect on growth. *Error bars* represent standard errors

sis. Figure 15.4 shows that even lower doses of ATPγS and ADPβS, in the 10–20 µM range, can stimulate hypocotyl growth, while doses above 400 µM inhibit growth. Comparing these results with the growth results shown in Tang et al. (2003), we find that the threshold for inhibiting growth is about 10 times lower when the poorly hydrolyzable nucleotides are applied. Taken together, the data show that nucleotide hydrolysis is not required for its growth effects, and suggest that the less sensitive responses to ATP and ADP may be due to the rapidity with which these nucleotides are hydrolyzed in the plant cell wall, where acid and alkaline phosphatases abound.

To relate the previously mentioned results to physiology, Coco et al. (2003) proposed that sites of active delivery of secretory vesicles are sites of release of high concentrations of ATP, because secretory vesicles contain concentrations of ATP near millimolar (Pugielli et al. 1999), and they would unload this cargo of ATP into the ECM when they fuse with the plasma membrane. If this is true also in plants, then one would predict that the growth of plant cells would be accompanied by the delivery of ATP to plant cell walls, because sites of active growth in plants are also sites of active delivery of secretory vesicles (Clark et al. 2005).

Since high levels of ATP can inhibit growth, one could also postulate that control of eATP concentration at growth sites would be a mechanism of growth control. Consistent with this hypothesis, treatment of wild-type pollen with either micromolar levels of ATPγS or with inhibitors of the ATP-hydrolyzing enzyme apyrase inhibits pollen germination (Steinebrunner et al. 2003). Knocking out AtApy1 and AtApy2, two closely related apyrases

that are both strongly expressed in wild-type pollen, also blocks pollen germination (Steinebrunner et al. 2003). However, although it is likely that *Arabidopsis*, like peas (Thomas et al. 1999) and like animals, has ectoapyrases that control the extracellular concentration of ATP, the subcellular locale of AtApy1 and AtApy2 in *Arabidopsis* has not been confirmed, so their regulation of pollen germination may or may not be through their maintenance of a growth-promoting level of eATP concentration in pollen walls. By sequence analysis there appear to be seven different apyrases in *Arabidopsis*, and it will be important to determine which of these function as ectoapyrases, and thus as potential regulators of extracellular nucleotide agents that may serve as growth regulators in plants.

Legume apyrases have been strongly implicated in the process of Nod signaling (Cohn et al. 2001), and they also appear to play a role in plant defense against pathogens (Kawahara et al. 2003). Although these enzymes have not been a focus of this review, to the extent that members of this family control eATP concentration they could obviously play major roles in growth control. Progress in defining the role(s) of apyrases in nodulation will be synergistic with progress in defining the role(s) of apyrases in growth control in *Arabidopsis* and other non-nodulating species. Quite likely, the apyrase studies in legumes may also catalyze more rapid progress in defining the role(s) of extracellular nucleotides in plant growth and development.

15.4 Conclusions and Future Perspectives

The data at hand strongly favor the conclusion that extracellular nucleotides can induce signaling changes in plant cells that are potentially growth regulating. A key limiting factor for future progress in this exciting new field is the lack of a clearly identified P2-like purinoceptor. The primary structure of these receptors is not highly conserved even in mammals, and a close homolog of the mammalian P2 purinoceptors has not been identified either in plants or in other animals like *Drosophila* or *Caenorhabditis elegans*. However, Demidchik et al. (2003) hinted that they were making progress on this front, and if they are successful in identifying a receptor for extracellular nucleotides, this would represent a major advance.

Another key area critical for future progress is defining the mechanisms and physiological situations that promote the release of nucleotides to the wall of plant cells and that control their concentration there. A prerequisite for this progress will be developing techniques for rapidly and quantitatively measuring changes in the concentration of extracellular nucleotides in plant cell walls. Progress in this area has been relatively slow even in

animal cells, although several works have used luciferin–luciferase-based reporters localized to the ECM to obtain initial data on this critical measurement (Joseph et al. 2003), and plant scientists are testing this approach also in plants (Gary Stacey, University of Missouri, personal communication).

If and when a purinoceptor is identified in plants and methods for measuring changes in eATP concentration are perfected, the field of nucleotide signaling will rapidly expand in significance, as will the field of ectoapyrase studies. Since virtually all growth-signaling pathways are interlinked, future breakthroughs on the impact of extracellular nucleotides on plant growth and development will also inform diverse other fields that focus on growth control (e.g., hormone physiology, photomorphogenesis, gravitropism/phototropism, plant reproduction). The field of extracellular nucleotide signaling in plants is in its infancy, and we expect the most exciting insights are yet to come.

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16 Physiological Roles of Nonselective Cation Channels in the Plasma Membrane of Higher Plants

Vadim Demidchik

Abstract Nonselective cation channels (NSCC) in the plasma membrane of higher plants form a large and diverse group of plant cation channels which are permeable for K^+, Na^+ and Ca^{2+}. They include four classes: (1) constitutive NSCC; (2) NSCC activated by reactive oxygen species (ROS); (3) ligand-activated NSCC; and (4) mechanosensitive NSCC. Our understanding of physiological functions of NSCC has significantly progressed in the last few years. NSCC were demonstrated to be involved in nutritional uptake of K^+, NH_4^+, Ca^{2+}, Mg^{2+}, micronutrients and trace elements, toxic Na^+ influx, in ROS-, amino acid, purine- and cyclic nucleotide induced signalling, growth and development. Here, physiological roles of plant NSCC are reviewed and discussed.

16.1 Introduction

Plant ion channels transporting cations through the plasma membrane can be divided in to three main groups. The first identified group is K^+ channels that includes two large classes, namely outwardly and inwardly rectifying K^+ channels (KOR and KIR respectively) (reviewed by Very and Sentenac 2003). These channels are highly selective for K^+ and NH_4^+ over other cations and are involved in K^+ and NH_4^+ uptake, release and redistribution in plants. The second group of cation channels is Ca^{2+} channels. This group embraces depolarisation-activated (Thuleau et al. 1994; Thion et al. 1996) and hyperpolarisation-activated (Gelli and Blumwald 1997; Kiegle et al. 2000; Very and Davies 2000; Demidchik et al. 2002) Ca^{2+} channels (DACC and HACC respectively). Cation selectivity of Ca^{2+} channels has been properly tested only in a few studies; therefore, it is unclear whether these channels are highly selective for Ca^{2+} over all other cations (like the animal Ca^{2+} channel) or not. Besides, genes-encoding classic voltage-gated Ca^{2+} channels (known from animal physiology) have not been identified in plants (White et al. 2002). So, many plant Ca^{2+}-permeable channels very likely belong to nonselective cation channels (NSCC). NSCC form the third group of plant cation channels with about equal permeability for K^+ and Na^+ ($P_K/P_{Na}<3$) and high Ca^{2+} permeability ($P_{Ca}/P_K > 0.1-0.2$) (Demidchik et al. 2002). The focus of this review is on NSCC with a particular emphasis on their physiological functions in plants. Our understanding of the physiological roles of NSCC has improved significantly, but not much

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progress has been made with respect to molecular characterisation of candidate genes for NSCC since the first putative plasma membrane NSCC were discovered in plants (Lam et al. 1998).

16.2
Physiological Roles of Animal NSCC

In animals, NSCC are a diverse group of channels with different structures and physiological roles (Hescheler and Schultz 1993). A modern NSCC classification has been published on the web (http://www.neuro.wustl.edu/neuromuscular/mother/chan.html). These channels play a major role in recognition of hormones and neurotransmitters and catalyse early cellular responses to these substances. NSCC are involved in volume regulation, sensing H^+, Ca^{2+}, reactive oxygen species (ROS) and intracellular cyclic nucleotides. Animal glutamate-, purine-, cyclic-nucleotide-, and acetylcholine-activated ionotropic receptors are NSCC. They mediate communication between cells and establish a basis for a high nervous activity. Depolarisation (due to K^+ and Na^+ fluxes) and/or increase in the cytosolic Ca^{2+} activity ($[Ca^{2+}]_{cyt}$) are frequently consequences of activation of NSCC at the cellular level. These NSCC-mediated effects can directly or indirectly (through the second messengers and changes in gene expression) alter physiological processes at the organismal level.

16.3
Functional Classification of Plant NSCC

Plant NSCC exist both in the plasma membrane and in the tonoplast. Tonoplast NSCC are well studied at the physiological level in many plant species (reviewed by Demidchik et al. 2002). Plasma membrane NSCC embrace (1) constitutive NSCC, (2) ROS-activated NSCC, (3) ligand-activated NSCC, and (4) mechanosensitive NSCC. Constitutive NSCC are active permanently without activating factors. Ligand-activated NSCC open after interaction with a specific chemical substance (ligand). ROS-activated and mechanosensitive NSCC require the presence of ROS or stretching for activation, respectively. All these channels vary in biophysical properties (reviewed by Demidchik et al. 2002).

16.4 The Role of NSCC in Plant Mineral Nutrition

Uptake of cations is a crucial physiological process that is involved in plant cell metabolism, photosynthesis, productivity and resistance to stresses (Bergmann 1992; Marschner 1995). Four macronutrients can be taken up by roots in cationic form – N, K, Ca, and Mg (as NH_4^+, K^+, Ca^{2+} and Mg^{2+}). Most micronutrients are cations that can potentially permeate NSCC (Na^+, Fe^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+}). With the exception of K^+ and NH_4^+, passive influx of cations in plant roots is probably dominated by the activity of NSCC. Cations can be transported into the cytosol by hyperpolarisation-activated and voltage-insensitive NSCC that are functional at voltages allowing passive cation influx. Depolarisation-activated NSCC are unlikely to play significant roles in the influx of monovalent cations, but they could transport divalent cations (because the activity of divalent cations in the cytosol is low and they cross the plasma membrane passively at positive membrane potentials).

16.4.1 Potassium and Ammonium

Many constitutive NSCC are permeable to K^+ (reviewed by Demidchik et al. 2002). With few exceptions, for example, NSCC in pea leaf epidermis (Elzenga and van Volkenburgh 1994) and maize root cortex (Roberts and Tester 1997), K^+ permeates through NSCC better than other cations. Permeability to NH_4^+ has been measured in several preparations (Cerana and Colombo 1992; Demidchik and Tester 2002; Roberts and Tyerman 2002; Zhang et al. 2002), often with a slightly lower permeability than for K^+ $P_{NH_4^+}/P_K \approx 0.7$. However, a slightly greater selectivity for NH_4^+ over K^+ was found in the symbiosome membrane of *Lotus japonicus* (Roberts and Tyerman 2002). In this membrane NSCC probably transport fixed NH_4^+ from *Rhizobia* bacteria and the legume host.

According to Hirsch et al. (1998) the $^{86}Rb^+$ uptake rate was about 10, 5 and 2 times higher at 10, 100 and 1,000 μM external Rb^+ respectively in wild-type than in knockout lacking inwardly rectifying K^+ channels (*akt1*). So, the role of KIR in K^+ uptake is very high at moderately low external K^+ concentrations but, under average soil K^+ concentrations (0.1–1 mM; Bergmann 1992) NSCC could mediate about 20–35% of total accumulated K^+. Many KIR are highly permeable for NH_4^+ but not all of them (Bertl et al. 1997); therefore, in some cases, passive NH_4^+ influx is likely mediated by NSCC (Kronzucker et al. 2001).

Ligand-activated and ROS-activated NSCC can potentially play an important role in the nutritional K^+ influx. Demidchik et al. (2004) have demonstrated that glutamate-activated NSCC in *Arabidopsis* root epidermis catalyse significant K^+ influx currents. High K^+ permeability of glutamate-activated NSCC is probably responsible for a glutamate-induced depolarisation of the plasma membrane in *Arabidopsis* root cells (Dennison and Spalding 2000). $OH^•$-activated NSCC were shown to mediate large K^+ and NH_4^+ influx currents (Demidchik et al. 2003). Mechanosensitive NSCC in *Allium cepa* bulb epidermis are well permeable to K^+ (Ding and Pickard 1993) but their role in nutritional K^+ uptake is difficult to predict owing to very limited data on this group of channels.

16.4.2 Calcium and Magnesium

Extracellular Ca^{2+} and Mg^{2+} concentrations are similar (between 0.1–2 mM) but $[Ca^{2+}]_{cyt}$ (about 100 nM) is dramatically lower than cytosolic Mg^{2+} activity (0.4–2 mM)(Bergmann 1992; Marschner 1995). This results in very positive E_{Ca} (from 87 to 125 mV) and E_{Mg} about 0 mV. At negative membrane potentials, both ions can be passively transported to the cell but only Ca^{2+} can pass a membrane through the channels at positive potentials.

According to Romano et al. (1998) depolarisation-activated K^+-permeable channels in *Arabidopsis* mesophyll protoplasts can catalyse a significant Ca^{2+} influx. Similar results were found in root protoplasts (Roberts and Tester 1997; Gilliham et al. 2004). Selectivity series for single Ca^{2+}-permeable outwardly rectifying conductances in the maize root showed that they were catalysed by NSCC (Roberts and Tester 1997). So, depolarisation-activated NSCC could catalyse some Ca^{2+} influx.

Passive transport of Ca^{2+} for nutritional needs is a key question of plant mineral nutrition (Marschner 1995). HACC activate at membrane potentials more negative than −150 mV (Véry and Davies 2000). When $[Ca^{2+}]_{cyt}$ increases HACC activate at more positive voltages and therefore probably play a significant role in Ca^{2+} uptake in growing tissues (where $[Ca^{2+}]_{cyt}$ is elevated) (Véry and Davies 2000; Demidchik et al. 2002). DACC have low amplitude and are only functional in about 35% of protoplasts (Thion et al. 1996). DACC reveal maximal activation at −70 to −80 mV and could be involved in signalling, having no significant impact on nutritional Ca^{2+} influx. Typically plasma membrane potentials in *Arabidopsis* root epidermal cells vary between −101 and −144 mV (Maathuis and Sanders 1993; Demidchik et al. 2002), thus HACC or DACC cannot be a major Ca^{2+} influx pathway in these conditions. A key role of constitutive Ca^{2+}-permeable NSCC in nutritional Ca^{2+} influx has recently been demonstrated in *Arabidopsis*

root epidermis (Demidchik et al. 2002). These channels were clearly dominant in Ca^{2+} influx currents at resting membrane potentials. $^{44}Ca^{2+}$ flux measurements showed that the Ca^{2+} uptake rate was not sensitive to verapamil (HACC blocker) but was inhibited by Gd^{3+} (nonspecific blocker of cation channels). In addition, basal $[Ca^{2+}]_{cyt}$ revealed linear voltage dependence (NSCC have linear current–voltage relationships) that again implicates NSCC in Ca^{2+} uptake. Constitutive Ca^{2+}-permeable NSCC were permeable to Mg^{2+}, and could therefore participate in nutritional Mg^{2+} influx into the root.

Glutamate-activated Ca^{2+} influx channels have recently been identified in *Arabidopsis* root epidermal protoplasts (Demidchik et al. 2004). Glutamate-activated Ca^{2+} conductance appeared as "spiky" currents in about 20% of protoplasts. Protoplasts isolated from roots of aequorin-transformed *Arabidopsis* plants demonstrated steady-state (measured over 2 h) elevation of $[Ca^{2+}]_{cyt}$ in response to 0.2–2 mM glutamate, suggesting a significant role of glutamate-activated channels in the regulation of the basal cytosolic Ca^{2+} activity. Apoplastic glutamate concentration is between 0.3 and 1.3 mM, as measured in a range of tissues and species (Lohaus et al. 1995; Lohaus and Heldt 1997). Therefore, it is possible that apoplastic glutamate, and maybe glycine too (Dubos et al. 2003), functions as a "permanent" activator of Ca^{2+} influx into the plant cells.

Purine-induced Ca^{2+} influx has recently been discovered in *Arabidopsis* roots (Demidchik et al. 2003). Application of ATP and its nonhydrolysable derivatives resulted in a manifold transient increase in $[Ca^{2+}]_{cyt}$ fully recovered within 10–15 min after the application of purines. This suggests that, in contrast to glutamate, purines do not cause steady-state changes in Ca^{2+} influx and do not affect nutritional Ca^{2+} uptake.

Arabidopsis root and guard cell ROS-activated NSCC catalysed significant Ca^{2+} and Mg^{2+} influx that could play a role in nutritional uptake of these cations (Pei et al. 2000; Demidchik et al. 2003; Foreman et al. 2003).

16.4.3 Microelements and Trace Elements

It is generally accepted that Na^+ influx is catalysed by root epidermal NSCC (reviewed by Demidchik et al. 2002; Tester and Davenport 2003). Constitutive Na^+-permeable NSCC have been demonstrated in a range of tissues and species (Stoeckel and Takeda 1989; Elzenga and van Volkenburgh 1994; White and Lemtiri-Chlieh 1995; Tyerman et al. 1997; Roberts and Tester 1997; Amtmann et al. 1997; Véry et al. 1998; Demidchik and Tester 2002). In the majority of preparations these channels were weakly selective for monovalent cations, voltage-insensitive (or slightly voltage

dependent), blocked by lanthanides, insensitive to Ca^{2+} and K^+ channel inhibitors (such as nifedipine, verapamil and tetraethylammonium), and showed fast activation kinetics (in the millisecond range).

In addition to permeating constitutive NSCC, Na^+ can also permeate a membrane through glutamate-activated conductance, as was recently characterised in *Arabidopsis* root epidermis (Demidchik et al. 2004). Glutamate-induced Na^+ influx currents were present in a low proportion of protoplasts (about one tenth of the protoplasts at 0.1 mM and about one third at 3 mM glutamate), were weakly voltage dependent, permeable to K^+, Cs^+ and Ca^{2+} and rapidly activating. However, the role of Na^+ influx through glutamate-activated channels is obscure because $^{44}Na^+$ was only slightly affected by glutamate (Demidchik et al. 2004).

ROS-activated NSCC are permeable to Na^+ ($P_K/P_{Na} \approx 0.7$) (Demidchik et al. 2003). NaCl was shown to stimulate the production of extracellular ROS (Demidchik et al. 2003). I suggest that NaCl-induced ROS production can activate Na^+ uptake through ROS-activated cation channels. This mechanism can potentially be very important for toxic Na^+ influx under salinity.

Welch (1995) suggested that "nonspecific" cation channels are involved in the plant uptake of cation micronutrients and toxic metals, and since then a number of reports have been published about the NSCC-mediated influx of different metals in plants (Demidchik et al. 2002, 2003; Reid and Liu, 2004). The uptake of micronutrients, toxic and trace elements could be an important physiological function of NSCC. This function is based on NSCC domination in divalent cation influx at resting membrane voltages (Demidchik et al. 2002). Constitutive NSCC were shown to conduct large influx currents of Zn^{2+} ($P_{Ca}/P_{Zn} \approx 0.51$) (Demidchik et al. 2002). ROS-activated NSCC were also permeable to this cation (Foreman et al. 2003). Experimental evidence supporting the involvement of cyclic nucleotide-gated channels (CNGC) in transport of Ni^{2+} and Pb^{2+} has recently been reported for tobacco plants (Arazi et al. 1999; Sunkar et al. 2000). Reid and Liu (2004) have demonstrated that when extracellular radio-labelled Co^{2+} concentrations were between 1 and 10 μM this micronutrient was taken up into plants exclusively by NSCC. NSCC also play a crucial role in an accumulation of Chernobyl pollutant $^{37}Cs^+$ (White and Broadley 2000; Broadley et al. 2001; Demidchik et al. 2002).

16.5 The Role of NSCC in Plant Signalling

Figure 16.1 shows a model of possible NSCC involvement in plant signalling and communication processes. These processes are necessary for

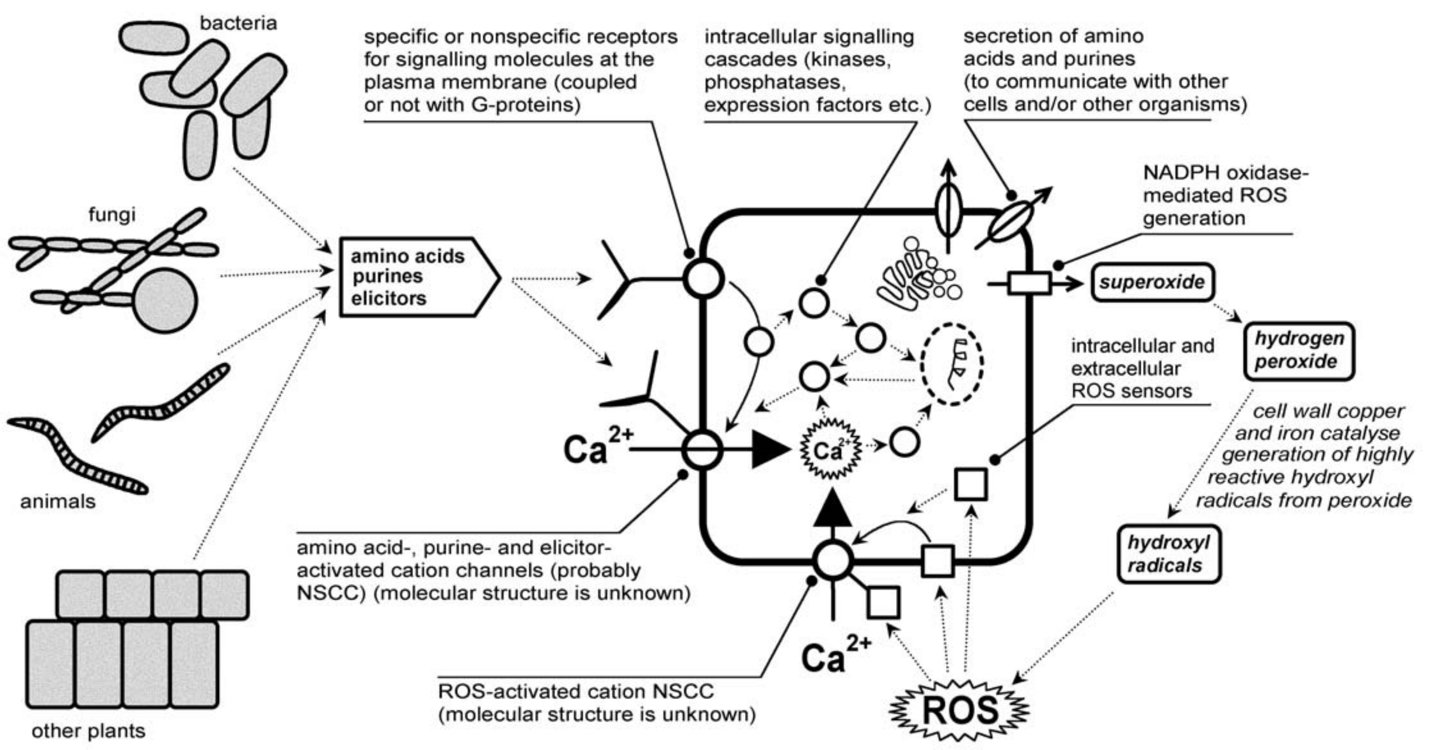

Fig. 16.1. Possible roles of nonselective cation channels (*NSCC*) in plant signalling

adaptation to new environments, stress, gravitropic and hormonal responses, interaction with pathogens, and coordinated behaviour of cells during growth and development. Ligand- and ROS-activated NSCC catalyse Ca^{2+} influx to the cytosol in response to signalling substances (ligands) that could result in generation of a transient increase in Ca^{2+} activity having signal-specific shape. Calcium transients probably trigger both signal-specific gene expression and direct Ca^{2+}-mediated changes in intracellular reactions. Here, I briefly describe ligand- and ROS-activated Ca^{2+}-permeable NSCC that have been found in plants.

ROS (hydrogen peroxide, superoxide and hydroxyl radical) are important signalling agents in plants that are involved in many physiological phenomena (Apel and Hirt 2004). Plant ROS-activated NSCC were found for the first time in intact cells of the freshwater alga *Nitella flexilis* (Demidchik et al. 1997, 2001). Extracellular Cu^{2+}, which can generate $OH^{\bullet}$ in intact cell walls (Fry et al. 2002), caused significant influx currents that were nonselective for monovalent cations, voltage-independent and instantaneously activating (Demidchik et al. 1997). Calcium permeability of ROS-activated NSCC was found for the first time in *Arabidopsis* guard cells (Pei et al. 2000). H_2O_2 activated significant Ca^{2+} influx currents that regulated stomatal closing. In contrast to the case for guard cells, H_2O_2 did not activate cation conductances in *Arabidopsis* root protoplasts, however $OH^{\bullet}$ was effective in this preparation (Demidchik et al. 2003; Foreman et al. 2003). *Arabidopsis* root $OH^{\bullet}$-activated channels were permeable for monovalent and divalent cations, showed inward rectification and slow kinetics, and were sensitive to lanthanides. They mediated an increase in $[Ca^{2+}]_{cyt}$ which could encode information about stresses (Demidchik et al. 2003).

Glutamate and glycine are animal neurotransmitters and important signalling agents regulating a number of physiological functions (reviewed by Dingledine et al. 1999). In plants, they can induce NSCC-mediated Ca^{2+} influx conductances, plasma membrane depolarisation and cytosolic Ca^{2+} transients (Dennison and Spalding 2000; Dubos et al. 2003; Demidchik et al. 2004), most likely through the activation of plant ionotropic glutamate receptors (Lam et al. 1998; Lacombe et al. 2001). Glutamate-activated Ca^{2+} transients were greater in the mature epidermis than in inner layers of root cells (Demidchik et al. 2004), suggesting that glutamate can be an external stimulus from rhizosphere (from other plants, pathogens and parasites). Apoplastic glutamate and glycine concentrations are at least 0.1–0.3 mM, as measured in *Arabidopsis* roots (Demidchik et al. unpublished), therefore they could play signalling roles at higher concentrations (about 1 mM). Systems excreting and breaking down apoplastic amino acids have not yet been identified in plants. Therefore, signalling roles of glutamate-/glycine-activated NSCC are very likely restricted by perception of external stimuli from other organisms (Fig. 16.1).

Molecular characterisation of plant glutamate receptors is in progress. Knocking out *AtGLR1.1* (one of *Arabidopsis* glutamate receptor homologues) showed a possible involvement of glutamate receptors in the seed germination, N and C nutrition, and in abscisic acid regulation of the root growth (Kang and Turano 2003; Kang et al. 2004). Changes in glutamate-induced cation fluxes have not been tested in *Atglr1.1*. Kim et al. (2001) have shown that *AtGLR2* could be involved in Ca^{2+} transport and utilisation in *Arabidopsis*. According to Turano et al. (2002) *AtGLR3.2* is expressed in growing tissues and vessels where it could be implicated in Ca^{2+} transport. Phylogenetic and expression analyses of the *Arabidopsis* glutamate-receptor-like gene family suggest a particular role of glutamate receptors in the plant root physiology because all 20 *AtGLR*s were expressed in roots (Chiu et al. 2002).

Cyclic nucleotides (cAMP and cGMP) are ubiquitous signalling molecules in all living organisms. CNGC are thought to be a target for these signalling agents (Talke et al. 2003). Genes encoding CNGC have been identified in plants (reviewed by Demidchik et al. 2002). Knocking-out *AtCNGC2* (*dnd1*) and *AtCNGC4* (*hlm1*) suppressed hypersensitive response and altered resistance to pathogens (Clough et al. 2000; Balague et al. 2003; Jurkowski et al. 2004). This suggests that CNGC participate in plant Ca^{2+} transport. Indeed, heterologously expressed plant CNGC demonstrate activation by cyclic nucleotides and permeability to monovalent and divalent cations (Leng et al. 1999, 2002). In *Arabidopsis* guard cells and mesophyll, Lemtiri-Chlieh and Berkowitz (2004) have recently characterised Ca^{2+}-permeable CNGC with inward rectification and sensitivity to lanthanides. However, Maathuis and Sanders (2001) reported an inhibitory effect of cyclic nucleotides on constitutive Na^+-permeable NSCC in *Arabidopsis* root-derived protoplasts. Therefore, the precise role of CNGC in the plant signalling remains obscure.

Purines play a central role in energy metabolism: however, in animals, they also function as signalling molecules activating ionotropic purinergic receptors (which are NSCC) (Ralevic and Burnstock 1998). In plants, ABC transporters could release ATP and ADP to the extracellular space, whilst ectoapyrases could break these molecules down (Thomas et al. 1999). These systems can potentially provide rapid release/removal of purines that is necessary for purinergic signalling (Ralevic and Burnstock 1998). Does purine signalling exist in plants? Three recent reports show that such signalling may exist in plants. Lew and Dearnaley (2000) have found that externally applied ATP and ADP depolarise the plasma membrane of *Arabidopsis* root hair. Demidchik et al. (2003) have demonstrated that different purines (including nonhydrolysable ATP analogues) induce transient elevations in $[Ca^{2+}]_{cyt}$. Jeter et al. (2004) have additionally shown that purines activate Ca^{2+}-mediated cytosolic signalling cascades. Purine-induced $[Ca^{2+}]_{cyt}$

transients were blocked by inhibitors of animal purinoceptors (suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid) (Demidchik et al. 2003). Overall, these data suggest that plants evolved systems for purine signalling which are very similar to animal purinoceptors.

16.6 The Role of NSCC in Plant Growth and Development

Despite a number of observations showing the indirect involvement of ligand-gated NSCC (reviewed by Davenport 2002; Talke et al. 2003) in plant growth and development, data demonstrating direct evidence for such involvement are very limited (Demidchik et al. 2002, 2003; Foreman et al. 2003). It was shown that elongation of cells in the elongation zone and elongation of root hairs is driven by an ROS/Ca^{2+}-dependent mechanism that is mediated by plasma membrane NADPH oxidase and ROS-activated Ca^{2+}-permeable NSCC (Demidchik et al. 2003; Foreman et al. 2003). In addition, constitutive NSCC were shown to have higher activity in the root elongation zone that could promote further $[Ca^{2+}]_{cyt}$ elevation through activation of HACC (Demidchik et al. 2002).

16.7 Conclusions and Future Perspectives

Our understanding of physiological roles of NSCC has significantly progressed in the last few years. Apart from toxic Na^+ influx, this group of channels was shown to be involved in nutritional uptake of K^+, NH_4^+, Ca^{2+}, Mg^{2+}, micronutrients and trace elements, in ROS-, amino acid, purine- and cyclic nucleotide induced signalling, growth and development. The next step in studying NSCC will be the delineation of their involvement in communication between plant cells, and between plants and other organisms. Another important task for future research is to find genes encoding plant purinoceptors, ROS-activated NSCC and constitutive NSCC.

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17 Touch-Responsive Behaviors and Gene Expression in Plants

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Abstract The abilities of plants to perceive stimuli in their environment are often overlooked. Yet hints of exquisite sensitivities abound. Indeed, although it is not generally appreciated, probably all plants can perceive and respond to simple mechanostimulation like touch. We will briefly review some of the more spectacular touch responses of specialized plants and then discuss developmental and molecular responses to touch that occur in nonspecialized plants. Many of these data and concepts have recently been reviewed by Braam (*New Phytol.* 165:373–389, 2005).

17.1 Specialized Plants – Touch Responses That Catch Attention

Fast reactions are easy to see and therefore the touch responses of plants like Venus' flytrap, sundew and *Mimosa* are well recognized. Venus' flytrap (*Dionaea muscipula*) and sundew (e.g., *Drosera rotundifolia*) use touch-sensitive responses to carry out carnivory by cleverly trapping insects; these touch responses enable them to thrive in nitrogen poor soils. Venus' flytrap awaits potential prey by spreading wide its specialized bi-lobed leaves edged with needle-shaped tines (Fig. 17.1a). Trigger hairs on the ventral leaf surface must be touch-stimulated multiple times to induce trap closure (Curtis 1834). Recent examination of the changes that take place in leaf geometry upon rapid closing reveal that the rapid closure comes from a snap buckling of the leaves as they transition from open to closed state (Forterre et al. 2005). The signaling that must occur between the trigger hairs and the closure response is still somewhat mysterious; however, intercellular electrical changes are detected and may act as intermediary signals (Burdon-Sanderson 1873; Jacobs 1954; Jacobson 1965; Simons 1981; Fagerberg and Allain 1991).

The sundew attracts insects with its glistening mucilage-laden tentacles (Fig. 17.1b). Insects that alight upon the tentacle surfaces find themselves bound by the gluey mucilage. The agitated movements of the insect attempting to loosen itself from the sticky surface lead to touch-induced tropic and nastic movements of neighboring tentacles (Darwin 1893; Lloyd 1942). An indentation of the leaf generates a cup-shaped enclosure (Fig. 17.1b) where the meal is dissolved. The selective sensitivity of the tentacles is remarkable. Darwin (1880, 1893) reported that the tentacles are capable of

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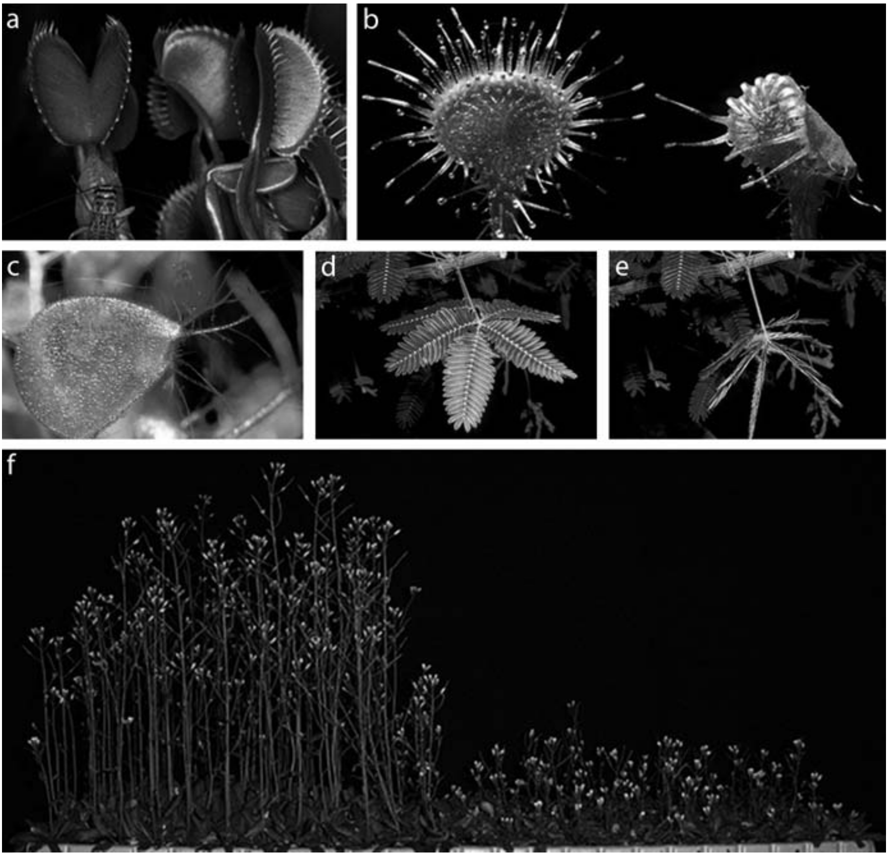

Fig. 17.1. **a** Potential prey nears a *Dionaea muscipula* (Venus' flytrap). **b** Open *Drosera* (sundew) leaf before and after touch stimulation. **c** *Utricularia inflata* (bladderwort) side view with tentacles visible at right near trap door. The doubly compound leaves of *Mimosa pudica* (sensitive plant) **d** open before stimulation and **e** closed after stimulation. **f** Repetitive touch stimulation leads to a delay in flowering and an inhibition of inflorescence elongation in *Arabidopsis*. The plants on the *right* were touched twice daily; the plants on the *left* are untreated controls. (Reproduced with permission of the New Phytologist Trust; Braam 2005)

detecting a strand of human hair weighing less than a microgram and yet rain droplets have little effect in activating the movements.

The trap of the bladderwort (*Utricularia*) may operate with similar mechanisms described recently for Venus' flytrap (Forterre et al. 2005). This aquatic rootless plant uses a thin-walled hollow sac and a watertight trapdoor as its prison and digestion chamber (Fig. 17.1c) (Lloyd 1942). The outer walls are curved inward, pulled perhaps by the negative hydrostatic pressure inside the bladder. Touch-sensitive appendages extend out near the trapdoor. When these triggers sense touch, by waterfleas or other small

creatures that have ventured close to the bladder opening, the door bursts open within 30 ms and water, and any unfortunate creatures caught in the current, rushes the chamber as the outer walls flip from concave to slightly convex in shape.

The touch-induced leaflet movements of the sensitive plant (*Mimosa pudica*) are more likely to occur for protective rather than aggressive purposes and adjacent, but untouched, leaflets fold up (Fig. 17.1d,e). If all the leaflets of the compound leaf undergo this movement, as in response to a strong mechanical stimulation such as wounding, the whole leaf nearly disappears (Simons 1981; Malone 1994). Electrical, hydraulic and chemical signals have all been implicated in carrying the long-range information that enables responses even in unwounded leaves (Ricca 1916; Houwinck 1935; Simons 1981; Fromm and Eschrich 1988; Malone 1994; Fleurat-Lessard et al. 1997; Stahlberg and Cosgrove 1997).

17.2 Thigmotropism – Vines, Tendrils and Roots

Vines, tendrils and roots show expert thigmotropic behaviors. The *Monstera* vine uses both darkness and touch as growth and differentiation signals. The young vine searches the forest floor for darkness and grows toward dark shadows, a behavior called skototropism, to reach the base of its chosen host tree (Strong and Ray 1975). Upon touching the tree, the vine turns to grow upward and undergoes morphogenetic alterations such as leaf development and expansion as it ascends (Strong and Ray 1975).

Tendril coiling also enables plants to reach sunlit heights that would otherwise require the expensive generation and maintenance of a tall supporting trunk. The touch-sensitive tip coiling behaviors of tendrils begin within seconds to minutes; not as fast as the trapping responses described before, but with a time frame that selectively enables winding around and attachment to stable objects perceived in the local environment (Jaffe and Galston 1968). Indeed, tendrils that respond to transient touch stimulation are able to reverse their behavior by unwinding (Jaffe and Galston 1968). Tendrils are highly sensitive, being able to perceive stimuli of a few milligrams or less (Darwin 1906; Simons 1992). In addition, tendrils are capable of distinguishing potentially productive and nonproductive perturbations; tendrils fail to coil in response to water droplets (Jaffe and Galston 1968). Tendrils can be thigmotropic, displaying coiling in a direction dependent upon the site of stimulation, or thigmonastic in that the direction of the coiling is predetermined and the touch stimulation simply triggers the response (Jaffe and Galston 1968). Octadecanoids and auxin

(indole-3-acetic acid) have been implicated as mediators of the differential growth effecting coiling (Jaffe and Galston 1968; Jaffe 1985; Weiler et al. 1993, 1994; Stelmach et al. 1998; Blechert et al. 1999).

Root tips are also highly sensitive to touch stimulation; perception of touch is thought to enable roots to avoid obstacles as they penetrate soils. Darwin (1880) and more recently Massa and Gilroy (2003) have observed that when root tips encounter an impenetrable surface such as a glass plate, the root tip flattens and turns 90 °C to grow over the surface until it is once again allowed to display positive gravitropism and turn to grow downward. Remarkably, perception of touch appears to delay the gravity-induced downward movement of columella cell starch granules and in this way may interfere with root gravitropism (Massa and Gilroy 2003).

17.3 Thigmomorphogenesis – Plasticity of Shoot Growth

Not only do the roots of nonspecialized plants sense and respond to touch, but the shoots are also mechanosensitive and mechanoresponsive. Mechanical perturbations, like touch or wind, generate gradual morphogenetic alterations in most, if not all, plants (Jaffe 1973). Plants subjected to repetitive mechanical stimuli develop with shorter and often stockier phenotypes (Fig. 17.1f). These changes occur slowly over time and therefore are not often recognized, but they can result in quite dramatic morphogenetic alterations. Jaffe, who conducted much of the pioneering work on this phenomenon, called the touch-induced changes in growth "thigmomorphogenesis" (Jaffe 1973). Thigmomorphogenesis likely evolved as an adaptive response to environmental stresses like wind and often results in increased rigidity or increased flexibility, depending upon the species, and may therefore improve resistance to further mechanical perturbation (Jaffe et al. 1984; Biddington 1986; Telewski and Jaffe 1986; Depege et al. 1997; Coutand et al. 2000).

Mechanical perturbation, like wind or touch, is likely perceived through the resulting longitudinal strain experienced by the shoot tissue. The extent of thigmomorphogenetic changes correlates strongly with the degree of longitudinal strain (Coutand et al. 2000). Furthermore, because thigmomorphogenetic alterations can affect subsequent strain, there are likely direct feedback pathways at play. The degree of thigmomorphogenesis would thus be tailored appropriately to the degree required for acclimation to the condition. For example, tobacco plants engineered to have weakened xylem composition would be predicted to have reduced tensile stiffness and should experience greater strain than wild-type plants. However, these transgenic tobacco are nearly indistinguishable from the wild-type control

in overall stiffness because the transgenics generate additional xylem tissue as apparent compensation for the material defects (Hepworth and Vincent 1999). These experimentally induced conditions are probably closely related to those that occur during natural progressive plant growth. Increased tissue strain would undoubtedly occur as plant size increases if there were no accompanying compensatory changes. Indeed, increased height in *Arabidopsis* is correlated with enhanced xylem production (Ko et al. 2004). The tissue alterations are likely induced by growth-associated strain increases. Consistent with these ideas, enhanced cambium differentiation can be induced by applying weight to immature *Arabidopsis* inflorescences (Ko et al. 2004), and cultured callus cells differentiate with cambium-like characteristics when subjected to compressive forces (Lintilhac and Vesecky 1984; Barnett and Asante 2000). Similar compensatory changes likely occur at a cellular level and can be observed when cell wall composition is altered by either mutation or inhibitors. Reduction in cellulose leads to enhanced pectin deposition (His et al. 2001); loss of lignin results in overaccumulation of cellulose (Hu et al. 1999). Thus, plants likely use strain as a measure of structural integrity at both the tissue and the cellular levels and can activate pathways that reinforce walls, tissues or organs in compensatory manners. The mechanosensory pathway is therefore undoubtedly critical for overall plant growth and development.

17.4 Mechanosensitive Gene Expression

What are the growth alterations that result in thigmomorphogenesis and how are they triggered? One clue may come from the dramatic changes in gene expression documented to occur very rapidly in plants subjected to mechanical perturbation. The first touch-inducible genes were discovered rather unintentionally (Braam and Davis 1990) and since then many genes have been found to show mechanosensitive expression regulation (reviewed in Lee et al. 2005). Microarray technology permits a deliberate approach to investigating the prevalence of touch-inducible genes in the plant genome. The Affymetrix chip (Affymetrix, Santa Clara, CA, USA) for the *Arabidopsis thaliana* genome enables the probing of 22,810 genes for inducible expression. Over 2.5% of the total, 589 genes, are upregulated in expression at least twofold within 30 min of a touch stimulation (Lee et al. 2005). Expression of 171 genes is reduced (Lee et al. 2005).

In addition to many genes of unknown function, genes encoding potential calcium (Ca^{2+})-binding proteins, cell wall synthesis and modification enzymes, protein kinases, transcription factors and disease-resistance proteins are most highly enriched in representation among those with twofold

or greater touch-inducible expression (Lee et al. 2005). Because calmodulin (CaM) and CaM-like proteins were among the first touch-inducible genes discovered (Braam and Davis 1990), it was not unexpected that these would be among those identified by the microarray as touch-inducible. Overall there is a 3.3-fold enrichment of Ca^{2+}-binding protein encoding genes among the genes upregulated at least twofold by touch (Braam and Davis 1990). Furthermore, Ca^{2+} has been implicated as a second messenger in mechanosensory signaling (Batiza et al. 1996; Calaghan and White 1999). Touch and wind trigger rapid cellular Ca^{2+} increases in plants (Knight et al. 1991). Thus, expression upregulation of Ca^{2+}-binding protein genes may be the result of a feedback pathway designed to produce more Ca^{2+} receptors when stimuli that use Ca^{2+} as a second messenger are perceived. Furthermore, because these proteins are potential Ca^{2+} sensors (McCormack and Braam 2003), they may function to transduce Ca^{2+} signals into cellular responses through Ca^{2+}-dependent changes in target protein activities. The microarray and subsequent real-time PCR data indicate that expression levels of at least one *CAM* (*CAM2*) of the seven *CAM*s and 14 of the 50 *CML*s are significantly upregulated by touch stimulation (Lee et al. 2005). The physiological functions of these touch-inducible genes encoding potential Ca^{2+} sensors remain largely unknown. However, transgenic plants with reduced CML24/TCH2 protein accumulation are defective in germination and seedling responses to abscisic acid, day-length regulated transition to flowering and growth inhibition induced by various salts (Delk et al. 2005).

Thigmomorphogenesis and the accompanying changes in structural properties would be predicted to require cell wall modifications. Cell walls are a major determinant of plant tissue integrity and form and thus changes occurring in thigmomorphogenesis likely involve alterations to the wall. Indeed, genes encoding cell wall synthesis and modification enzymes are among those most highly represented in the group of touch-inducible genes, undergoing a 2.5-fold increase in enrichment among the touch-inducible genes (Lee et al. 2005). Thus, touch-inducible expression of cell-wall-associated enzymes may underlie mechanostimulus-evoked morphogenetic changes.

Transcription factors and protein kinases are regulatory proteins whose production levels are often themselves regulated in response to diverse stimuli. Since these proteins can act as cellular switches to control physiological changes, their genes are typically among those most highly altered in expression in microarray experiments. Indeed, the touch-inducible gene cluster includes approximately twofold enrichment in both transcription factor and protein kinase genes (Lee et al. 2005).

Perhaps more unexpectedly, a major class of genes upregulated in expression by touch includes those that have been implicated in disease resistance (Lee et al. 2005). There is some evidence that suggests that

repetitive mechanical stimulation can lead to enhanced disease resistance (Biddington 1986); however, the basis of this potential resistance is not well understood. Notably a number of the disease-resistance-related genes upregulated in expression by touch are members of the nucleotide binding site (NBS) leucine-rich repeat (LRR) gene family. This family contains the majority of plant disease-resistance genes (R genes) identified to date (Cannon et al. 2002). The NBS domain is thought to contribute in signal transduction, and the LRR domain may be responsible chiefly for elicitor recognition (Cannon et al. 2002). The upregulation of expression of these genes suggests a potentiation of defense in touch-stimulated plants. Whether this potentiation results in an enhanced resistance response by plants is still to be determined.

It is also intriguing that many of the touch-inducible genes have been associated with the jasmonic acid (JA) pathway. In addition to genes encoding enzymes involved in JA biosynthesis, other touch-inducible genes are those that have been used as JA-dependent resistance markers, including those encoding proteinase inhibitors that block digestive enzymes of some insect herbivores. Recent literature indicates that plant defense responses against insect herbivores and some microbial pathogens are coordinated by JA signaling pathways (Howe 2001). JA-mediated defenses are typically preceded by accumulation of JA in response to biotic stress (Wasternack and Hause 2002). Whether touch results in the accumulation of JA and/or the establishment of an enhanced disease resistance state awaits further study.

Touch-inducible genes provide important tools for investigating how plants perceive mechanical stimuli; however, to date, most genes found to be touch-inducible in expression are also upregulated in expression by other types of stimuli. The original *TCH* genes, encoding CaM2, CML12, CML24, and XTH22, are not only upregulated in expression by various forms of stimuli that have mechanical properties, such as touch, wind and wounding, but also by stimuli such as cold, heat, and darkness that, at least superficially, do not appear to be mechanical in nature (Braam 1992, 2000; Sistrunk et al. 1994; Xu et al. 1995; Polisensky and Braam 1996). To address the question whether all touch-inducible genes share this property of being upregulated by diverse stimuli, microarray experiments were done using darkness as a stimulus (Lee et al. 2005). More than half of all touch-inducible genes were also upregulated in expression by darkness, and the coregulation was most apparent in those genes with the greatest fold touch inducibility (Lee et al. 2005). Indeed, among the top 60 touch-inducible genes with at least tenfold upregulation, only four were not at least twofold upregulated in plants treated with darkness (Lee et al. 2005). All but three of the 68 genes that are most strongly upregulated by darkness are also touch-inducible (Lee et al. 2005). These findings are consistent with an

interpretation that darkness and perhaps the other stimuli that induce expression of the *TCH* genes are related in some way. One possibility is that all these stimuli share the property of causing mechanical perturbations and in this way trigger a common mechanosensory pathway that leads to gene upregulation of expression (Braam 2000).

If the diverse stimuli that trigger *TCH* upregulation of expression act through a common pathway, then one would expect that a single *cis* regulatory element would be responsible for conferring the complex expression regulation. Indeed, a 102 base-pair region located upstream of the *TCH4* transcriptional start site is sufficient to confer touch, darkness, cold heat and epi-brassinolide upregulation of expression to reporter genes (Iliev et al. 2002). Sequences within this region share some similarities to sequences identified as important for conferring cold and touch inducibility to the *CBF2* gene (Zarka et al. 2003). The mechanism by which these sequences may act to regulate gene expression is currently unknown.

Distinct methods have been used to monitor *TCH* expression during plant morphogenesis. *TCH*::reporter transgenics have been characterized in addition to the more direct methods of immunolocalization and reverse transcription PCR (Sistrunk et al. 1994; Antosiewicz et al. 1995, 1997; Xu et al. 1995; Delk et al. 2005). In general, *TCH* expression is enriched at sites that may be predicted to experience mechanical stress. TCH protein accumulates and/or *TCH*::reporter transgenes are expressed in the ruptured seed coat, branch points, the root–shoot junction, elongating hypocotyls and roots, and developing trichomes and silique abscission zones. In addition, plants subjected to enhanced weight on the inflorescence have increased *TCH2* and *TCH4* expression (Ko et al. 2004). These data indicate that *TCH* expression may not only be upregulated in response to externally applied mechanical perturbations such as touch, but also by mechanical forces that become manifest during normal plant morphogenesis.

17.5 Conclusions and Future Prospects

Plants perceive much more of their environment than is often apparent to the casual observer. Touch can induce profound rapid responses and more slowly acquired growth alterations. Rapid touch-induced plant movement in specialized plants is often associated with predation or protection. The speed of these responses is an essential component of the response in these situations. Plants that acclimate over their lifetime to touch or wind stimuli also undergo dramatic touch-induced changes, but these, at least overtly, occur slowly over time. Molecular responses in nonspecialized plants can, however, occur quite rapidly. In *Arabidopsis*, changes in gene expression are

seen within minutes after touch, and over 700 genes have altered transcript levels within 30 min. The prevalence of touch-responsive genes and the rapidity with which such changes in gene expression occur are indications that nonspecialized plants do possess capacity for rapid responses to touch.

Recent research reveals the types of genes upregulated by touch and implicate Ca^{2+} signaling, cell wall modification and disease resistance as potential downstream responses. Further work is needed to reveal the physiological relevance of these touch-induced changes in gene expression. Furthermore, touch-induced genes are powerful molecular tools for the dissection of the perception and response pathways that enable plants to perceive mechanical stimuli and manifest appropriate responses. Perception mechanisms and intercellular and intracellular transduction machinery and signals await future discovery.

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18 Oscillations in Plants

Sergey Shabala

Abstract Despite the fact that the rhythmical behaviour is a quintessential pattern of life itself, most researchers still treat oscillations in plants as some unwanted "physiological noise". In this review, an attempt is made to summarise recent progress in this area and highlight the paramount role of oscillatory processes in plant life. First, diversity and hierarchy of oscillations in plants are examined, then a general overview of oscillatory phenomena is given, with the main emphasis on the physiological role of oscillatory processes in plants. The areas covered include leaf and stomata movement, nutations, nutrient acquisition, growth and differentiation, photosynthesis and osmotic adjustment. A possible role for ultradian rhythms in timekeeping is also briefly discussed. The importance of ultradian oscillations is further illustrated by discussing their involvement in the encoding mechanism, mediating plant–environment interaction. Finally, advantages and principles of oscillatory control are considered in the context of plant physiology, with a major emphasis on feedback control and self-sustained oscillations, as well as on deterministic chaos and "strange" behaviour in plants.

18.1 Introduction

With a possible exception of plant movements (such as those for leaves or plant axial organs) and oscillations in the stomatal aperture, many plant physiologists still treat oscillations as some unwanted or physiologically unrelated "noise" (Giersch 1994). More recently, a breakthrough in understanding of the signalling role of Ca^{2+} in cell metabolism caused a vivid interest in calcium oscillations in stomatal guard cells, as reflected by a large number of excellent reviews (McAinsh and Hetherington 1998; Blatt 2000). The physiological role of other oscillations found in plant tissues and organs is yet to be fully revealed. In this review, an attempt is made to summarise the recent progress in this area and highlight the paramount importance of oscillatory processes in plant life. The major focus is on ultradian (fast) oscillations, in the minute range of time periods. This is largely due to the fact that, despite a significant interest and breakthrough in our understanding of mechanisms of circadian oscillations in plants (Webb 2003), "astonishingly little research effort is currently devoted to ultradian high frequency oscillations in plant biology" (Lüttge and Hütt 2004).

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18.2 Diversity and Hierarchy of Plant Oscillators

18.2.1 Spatial and Temporary Hierarchy

Oscillations in the plant kingdom cover an extremely broad range of periods (Fig. 18.1) and occur at various levels of plant structural organisation. Consequently, we are talking about *spatial* and *temporal* hierarchy of oscillations in plants.

Spatial hierarchy implies that oscillations are present at different levels of plant structural organisation, from the molecular level to that for the whole plant or even of plant ecosystem (Aschoff 1981). The physiological significance of such spatial hierarchy is that the oscillating process in one "compartment" is optimised for a certain range of conditions and is not interfering with other "compartments". A good example is stomata patchiness and oscillations in leaf photosynthesis (Sect. 18.2.2.2).

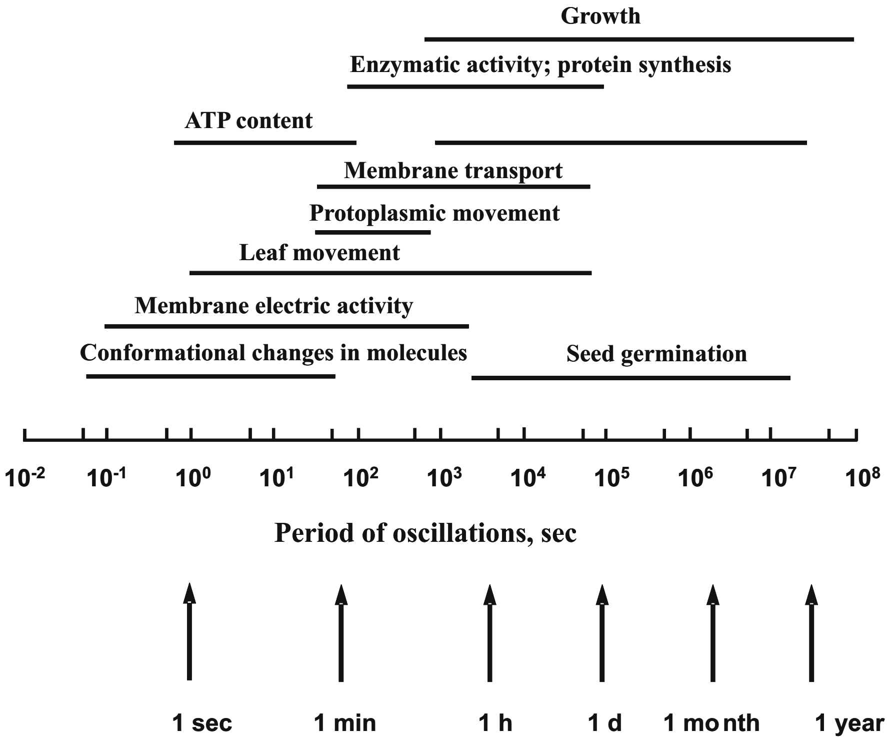

Fig. 18.1. Diversity and temporal hierarchy of plant oscillators

Temporal hierarchy implies that several oscillatory processes, with very different periods, may occur at the same cellular compartment, but because of the difference in the time scales in many cases one of these processes can be considered as stationary. The most apparent example is coexistence of circadian and ultradian oscillations for the same process. Oscillations in stomatal conductance (Barrs 1971; Cowan 1972) or leaf movement (Engelmann and Antkowiak 1998) are a good example. Another illustration may be net H^+ flux oscillations in the elongation zone of corn roots, with 7-min and 1.5-h period components (Shabala et al. 1997a).

The period range of reported oscillations in plants is extremely broad (Fig. 18.1), from a few milliseconds (oscillations in ion channel conductance; conformational changes in macromolecules) to several years (flowering cycles in some species) (Ashoff 1981; Shabala 1989, and references within). Most reported oscillations are between these two extremes (Fig. 18.1). These can be further divided into two major groups. One such group is circadian oscillations, with free-running periods between 20 and 28 h (Aschoff 1981; Webb 2003). The other (larger) group is oscillations in the minute range of periods. The latter group, the ultradian oscillations, is a major focus of this paper.

18.2.2 Functional Expression

Despite the ubiquitous presence of ultradian oscillations, their physiological role in plants is not always clear. Most published reports are phenomenological in nature, with no clear evidence for "functionality". In this section, I review some of the known evidence for ultradian oscillations in plants, with a major emphasis on the physiological significance of observed phenomena.

18.2.2.1 Leaf Movement

Oscillatory leaf movements, with periods from several minutes to 1–2 h have been reported for many species (Antkowiak and Engelmann 1995; Sharma et al. 2003). It is believed that reversible changes in leaf angle in plants bearing pulvini are caused by changes in the size and turgor of pulvini motor cells; these in turn are regulated by the rhythmical movement of K^+ and Cl^- into and out of such cells (Kim et al. 1993; Antkowiak and Engelmann 1995; Engelmann and Antkowiak 1998).

The functional role of ultradian oscillations in leaf movement is yet to be demonstrated in direct experiments. Several authors suggest that fast ultradian leaf movements may serve to regulate the light intensity, optimise

water and nutrient supply and reduce leaflet temperature by increasing transpiration (Engelmann and Antkowiak 1998; Sharma et al. 2003). The latter authors also reported that the period of oscillations in leaflet movement rhythm in *Desmodium* was strongly dependent on light intensity. Thus, these oscillations could serve the purpose of optimising the amount of light falling on the leaflet or/and facilitating transpiration of water through stomata.

18.2.2.2
Stomatal Control

Stomatal oscillations originate from several feedback loops regulating leaf water and gas status (Raschke 1975; Willmer 1988). This is undoubtedly the best-known class of ultradian oscillators in plants which have been known for a long time (Barrs 1971; Cowan 1972). These oscillations usually occur within a 10–90 min range (Raschke 1975) and are caused by abrupt changes of external parameters such as light, CO_2, O_2, H_2O and temperature (Giersch 1994; Luttge and Hutt 2004). Stomatal oscillations are an intrinsic feature of plant leaves, and the major reason these oscillations are not observed in every experiment is the fact that various leaf parts tend to oscillate out of phase (Siebke and Weis 1995). Ionic mechanisms of guard cell signalling have been the subject of numerous and comprehensive reviews (McAinsh and Hetherington 1998; Blatt 2000). The important question addressed here is: what are physiological implications of such oscillations?

Stomatal "patchiness" is a widely reported phenomenon (Cardon et al. 1994; Mott and Buckley 1998). Importantly, such "patchiness" is usually limited to a region bound by veins. The latter may be a result of a specific ionic (particularly, K^+) "microenvironment" in such regions, as revealed by correlating fine leaf anatomical structure and net K^+ and H^+ flux profiles (Shabala et al. 2002). Taken together, it may be suggested that such "small-scale" oscillations reported in plants (Siebke and Weis 1995) are required for "fine-tuning" of guard cell osmotic balance and optimising leaf photosynthesis for a specific light and ionic environment.

18.2.2.3
Nutations

Nutations of plant axial organs, with periods ranging from a few minutes to several hours, are found in both roots and shoots of a large number of species (reviewed by Barlow et al. 1994). These nutations are a result of differential growth, with membrane-transport processes being a likely "controlling mechanism" (Hejnowicz and Sievers 1995; Barlow et al. 1994; Shabala and Newman 1997). In nutating organs, cells on the convex side display a turgor pressure 10% greater than those of the concave side (Vanden

Driessche 2000). Recent experiments in our laboratory have found a strong ($R > 0.9$) correlation between root circumnutation patterns and rhythmic changes in K^+ uptake in vertically grown maize roots (Shabala 2003), with periodical ($t \sim 50$ min) K^+ flux changes from influx to efflux at two opposite sides in opposite phase.

Plant circumnutations show strong dependence on temperature ($Q_{10} \sim 2$; Schuster and Engelmann 1997), light intensity (Anderson-Bernadas et al. 1997) and quality (Schuster and Engelmann 1997), mechanical stimulation (Anderson-Bernadas et al. 1997) and chemical composition of the medium (Buer et al. 2000). All these observations point out the possibility of "encoding" environmental information by nutational patterns.

The traditional view of the physiological role of plant nutations is that nutational growth of an organ maximises the penetration of the medium in which it grows (Barlow et al. 1994). Recently, Inoue et al. (1999) showed that in rice root circumnutation was important in creating a larger pushing force of the seminal root without causing floating of the seedling on flooded and very soft soil, thus leading to a higher seedling establishment percentage in the field. More direct evidence is needed to fully reveal the role of nutations in plant axial organs.

18.2.2.4
Root Nutrient Acquisition

Despite the widely reported substantial diurnal changes in the rates of nutrient uptake by roots (reviewed by Shabala 2003), ultradian oscillations in root nutrient acquisitions remains underexplored. Nonetheless, such oscillations have been reported in several plant species (Knaritonashvili et al. 1997; Macduff and Dhanoa 1996; Shabala et al. 1997; Shabala and Knowles 2002; Shabala 2003), with periods ranging from 5 min to several hours. Importantly, such oscillations appear to be extremely sensitive to environmental conditions, with their periods showing a strong dependence on solution pH, temperature and osmolality (Shabala 2003). This may be strong evidence for a frequency-encoding mechanism operating in plant roots, similar to one reported for guard cells (McAinsh et al. 1995; Blatt 2000).

18.2.2.5
Growth

There is increasing evidence that axial growth of many plant organs is also rhythmically modulated. Ultradian oscillations in stem growth with periods of several minutes were reported for stems, hypocotyls, roots and sporangiphores (see Shabala 2003 for references). Oscillations in membrane-transport activity are likely to be the major driving force (Shabala et al. 1997a; Tyerman et al. 2001). It has been suggested that periodical fluctua-

tions in net H^+ efflux and K^+ uptake into the cell may be a useful strategy to balance cell wall loosening and turgor-driven cell expansion, given the observed phase shift between these two oscillatory cycles (Shabala 2003).

Another plant system exhibiting pronounced growth oscillations is pollen tubes. A pulsating growth of pollen tubes has been reported elsewhere (Feijo et al. 2001; Holdaway-Clarke and Hepler 2003). Both periodical changes in cell wall strength (Holdaway-Clarke and Hepler 2003) and rhythmical changes in cell turgor pressure (Messerli and Robinson 2003) are likely to control such oscillatory growth. The direct link and the specific mechanisms of growth oscillations and those in Ca^{2+}, H^+, K^+ and Cl^- fluxes from growing pollen tubes (Holdaway-Clarke and Hepler 2003) remain to be revealed.

18.2.2.6
Cell Differentiation and Morphogenesis

Another important function of ultradian cellular oscillations may be their involvement in cell differentiation and morphogenesis. There is no shortage of models suggesting that pattern formation in developing organisms may be the result of oscillatory dynamics (Jaeger and Goodwin 2001). Orientation of cell division and other cambial effects in trees was suggested to be a result of superposition of waves resulting from interacting cellular oscillators (Hejnowicz 1975). The idea that the ultradian clocks may control the cell division process is well established in microbiology (Lloyd and Stupfel 1991). It has been shown that the ultradian clock exerts control over energy-yielding processes, protein turnover, motility and the timing of cell-division processes in a large number of unicellular organisms (Lloyd and Kippert 1993). A key role of cyclic-AMP-dependent oscillations in the cellular differentiation processes of *Dictyostelium* was demonstrated elsewhere (Goldbeter et al. 1990).

For higher plants, direct experimental evidence is still lacking. Earlier we showed the evidence for large-amplitude ultradian Ca^{2+} flux oscillations in the meristematic region of corn roots (Shabala and Newman 1997). Unlike those in the elongation zone, these oscillations did not correlate with root nutational movement. It was suggested that such oscillations serve as a synchronising factor for cell division in the root meristem (Shabala and Newman 1997). More direct evidence is needed to verify this hypothesis.

18.2.2.7
Photosynthesis

In the natural environment, light is probably the most widely and rapidly fluctuating factor. During the course of a day, forest understorey plants are exposed to brief periods of high light superimposed on a low-light

background with a period ranging from a few seconds to 10 min (Pearcy 1990). On a larger scale, light fluctuations caused by cloud movements influence all plants growing in a particular area (Cardon et al. 1994), resulting in light fluctuations in the minute range of periods. It is not surprising, therefore, that leaf photosynthetic machinery is adjusted to such a "fluctuating" regime.

Oscillations in photosynthesis are well-reported phenomena (Kocks and Ross 1995; Lüttge and Hütt 2004) and have been found at various levels of organisation. Rhythmical changes in chlorophyll a fluorescence, phosphorylation, oxygen evolution and CO_2 assimilation are all examples of such oscillatory behaviour. The period range for such oscillations is usually around several minutes (Siebke et al. 1992). In most cases, such oscillations are strongly damped and, being induced by sudden changes in one of the environmental variables (light, CO_2, etc.), disappear after several cycles (Siebke et al. 1992; Kocks and Ross 1995). However, non-damped (for several hours) oscillations have also been reported (Siebke and Weis 1995).

18.2.2.8
Osmotic Adjustment

As discussed before, both leaf and axial organ movements are mediated by turgor and volume changes in the epidermal (in the case of nutations) or pulvini (in the case of leaf movement) cells. In addition, oscillatory ion transport mechanisms were shown to operate in planktonic diatoms for adjustment of buoyancy by appropriate uptake and release of ions (Gradmann and Boyd 1995). In higher plants, rapid (1–2 min period) cycles of K^+ uptake and release in osmotically stressed leaf mesophyll cells were reported (Shabala et al. 2000). All these facts point out the possibility of the "fine-tuning" mechanisms of osmotic adjustment being realised through oscillatory ion uptake across the plasma membrane.

18.2.2.9
Ultradian Rhythms in Time-Keeping

Molecular and genetics aspects of circadian rhythms have been the subject of recent reviews (Webb 2003). Transcriptional/translational genetics models are favoured (Dunlap 1998). Several clock genes have been identified (Webb 2003). However, it appears that circadian systems will almost certainly be made up of more than one interconnected feedback loop, and there are many discomforting facts for the current genetics model. Dunlap (1998) himself called it as a "pleasing caricature of reality".

One of the striking features of oscillations in plants is the coexistence of ultradian and circadian modulations of the same physiological process. Can ultradian rhythms be a part of the circadian clock mechanism?

The idea of the circadian clock being composed of a population of strongly coupled ultradian oscillators is rather old (Pavlidis 1971). According to this scenario, a 24 h rhythmicity can be achieved as a result of the frequency reduction ("beating") in an ensemble of interacting oscillators whose periods are much faster (in a minute range). The major drawback for all coupling models is the scale invariance. This problem was successfully resolved by suggesting the hierarchical coupling model (Barrio et al. 1996). If ultradian oscillators are assumed to be membrane-based, the temperature compensation problem may be achieved by temperature adaptation of membrane lipids as suggested in recent experiments on *Neurospora* (Lakin-Thomas 1998).

18.2.2.10
Oscillations as a Part of an Encoding Mechanism

The concept of frequency-encoded environmental information in plant cells has become rather popular among physiologists in relation to oscillatory Ca^{2+} spikes in guard cells (McAinsh et al. 1995; McAinsh and Hetherington 1998); however, it appears that this is also true for many other rhythmical processes in plants. Different environmental factors such as low temperatures, N and Fe starvation, mechanical stress, UV and salinity are known to modify characteristics of plant rhythms (Erdei et al. 1998). Cytosolic Ca^{2+} spikes in response to the *nod* factor were found in wild-type alfalfa plants, but not in non-nodulating mutant (Ehrhardt et al. 1996). Oscillations in root ion fluxes showed a clear dependence on solution pH, osmolality and nutrient availability (Shabala 2003). Answering the question about decoding mechanisms remains a great challenge for future research.

18.3
Advantages and Principles of Oscillatory Control

18.3.1
Feedback Control, Damping and Self-Sustained Oscillations

Why do plants (or plant components) exhibit oscillatory behaviour? The answer is rather simple: such behaviour is an intrinsic feature of every feedback-controlled system.

Virtually every aspect of plant metabolism is controlled by a large number of positive and negative feedback systems. Thus, it is *expected* that every physiological parameter in plants will oscillate, with some characteristic frequency (period), under certain conditions. What are these conditions?

The foundations of the *systems theory* suggest that if the relationship between several parameters within the system is described by linear equations, such a system should eventually reach its stable state (Stucki and Somogyi 1994). Once disturbed, such a system will eventually return to a new steady state through a series of damped oscillations. This is often observed for plant photosynthetic responses (Kocks and Ross 1995), changes in stomatal aperture (Barrs 1971) or cell electrophysiological characteristics (Tyerman et al. 2001).

The story is quite different if the system is governed by non-linear mechanisms. In that case, a limited cycle (a two-dimensional attractor), rather than a singular point, will be a stable condition (Stucki and Somogyi 1994). Thus, self-sustained oscillations are expected to be found in such non-linear systems.

There is no doubt that most physiological processes in plants are governed by non-linear mechanisms. Physiologically it means that within some narrow range of parameters (light intensity, ambient temperature, water and nutrient availability, etc.), plant responses might be linear. In this case, a sudden perturbation within this range will cause only a brief series of damped oscillations in plant physiological responses. As soon as the disturbance is beyond the range of the linear response, non-damping self-sustained oscillations are expected.

18.3.2
Advantages of Oscillatory Strategy

Every plant physiologist will probably agree that circadian rhythmicity in plants is a result of evolutionary adaptation of energy metabolism to optimise energy conservation with respect to daily environmental cycles of energy supply (Wagner et al. 1975). Researchers are less in agreement when discussing the functional role of ultradian oscillations in plants. Although there is no lack of theoretical investigations (see later) showing advantages of oscillatory strategy, direct evidence for plants is still rather rudimentary. Earlier Rapp (1987) described at least five positive functional advantages which periodic behaviour confers to living organisms. These are revisited next.

18.3.2.1
Temporal Organisation

Separate temporal compartments may be necessary where mutually incompatible biochemical reactions occur in an identical spatial (subcellular) compartment. Examples may include protein synthesis and degradation.

18.3.2.2
Spatial Organisation

Oscillations may provide synchronisation of events widely separated in space between different cells, or between subcellular compartments (Lloyd and Stupfel 1991). At the whole-plant level, frequency-coded signals are believed to play an important role in communication between the different organs and tissues (Wagner et al. 1998). Oscillations may also provide a specification of positional information during morphogenetical development (Hejnowicz 1975; Lloyd and Stupfel 1991).

18.3.2.3
Prediction of Repetitive Events

Oscillatory systems are able to respond rapidly to inputs received from their environmental surroundings (Lloyd and Stupfel 1991). A plant's ability to utilise sunflecks is one such example (Pearcy 1990). Also consistent with this idea, plant adaptation to a changing environment means resetting to a new circadian and/or ultradian rhythm (Erdei et al. 1998).

18.3.2.4
Efficiency

Theoretical models show that the energetic efficiency of oscillatory processes may be greater in the oscillatory mode than when steady-state behaviour prevails (Termonia and Ross 1982; Richter and Ross 1981), providing an evolutionary advantage over those organisms incapable of anything other than steady-state behaviour. Another advantage of an oscillatory strategy is that oscillations may enhance sensitivity to weak external stimuli (Dolmetsch et al. 1998).

18.3.2.5
Precision of Control

It is widely accepted that oscillations may act as a filter to discriminate true signals from environmental noise (Tsien and Tsien 1990; Lloyd and Stupfel 1991). Theoretical findings by Rapp et al. (1981) suggest that, being more robust to environmental perturbations, an oscillatory strategy provides significant functional advantages for living cells.

18.3.3
Deterministic Chaos and "Strange" Behaviour

Theoretical studies show that non-linear systems will possess complex dynamics leading to "strange" behaviour such as bifurcation and chaos (com-

prehensively reviewed by Lüttge and Hütt 2004). Termed as "deterministic chaos", such behaviour is routine in many physical and hydrodynamics systems. It still remains unclear, however, to what extent biological systems functionally exploit this behaviour (Lüttge and Hütt 2004).

Experimental evidence on chaos in plants is only gradually accumulating. The period doubling of leaf electric and temperature oscillations in response to rhythmical light (Shabala et al. 1986, 1997b), aperiodic leaflet movement reminiscent of homoclinic chaos (Chen et al. 1995) and chaotic behaviour in CO_2 exchange (Lüttge and Beck 1992) were reported. Chaotic oscillations in the plant cell expansion rate were predicted by Kellershohn et al. (1996), and bifurcational regimes in stomatal oscillations were modelled by Rand et al. (1981).

Such a dearth of experimental evidence of deterministic chaos in plants is more than surprising. Biological systems may gain benefits from exhibiting chaotic dynamics as it may contribute to the generation of diversity and hence adaptability (Lüttge and Hütt 2004). Advantages of chaotic dynamics were recently discussed (Lloyd 1997) and include greater flexibility in response towards external influences, phenotypic diversity, larger functional independence from external entrainment and higher dissipation of disturbance. Overall, exploitation of these benefits ensures the evolutionary survival of chaotic dynamics; thus, it can make "chaotic" behaviour in plants physiologically important.

18.3.4 Resonant Regimes

The *Dictionary of Physics* defines resonance as a "condition in which a vibrating system responds with maximum amplitude to an alternating driving force". Resonance phenomena are widely used in various areas of modern life (engineering, medicine, etc.). Keeping in mind the wide range of oscillatory activity in plants, we have to answer two questions: (1) are resonant responses possible in plants? and (2) will such behaviour be beneficial to plants?

The answer to both questions is "yes". There is no shortage of theoretical models predicting resonant plant responses to periodical disturbance. Examples include photosynthetic responses (Kocks and Ross 1995), membrane transport activity (Markevich and Sel'kov 1986; Tsong 1990) and energy transduction in glycolysis (Termonia and Ross 1982). These model predictions are further confirmed by experimental studies showing the resonant type of responses in stomatal conductance, water uptake and leaf surface electric potential to rhythmical light (Shabala 1989, 1997; Cardon et al. 1994) or root medium environment (Shabala et al. 1991). Preliminary

experiments suggested that seed germination rate, root regeneration ability and plant growth rate were all significantly increased when plants were grown under a resonant rhythmical light regime (Shabala et al. 1989; Shabala 1989). It is also worth mentioning that a 2.5-fold increase in ^{31}P uptake by plant roots was observed under these conditions (Shabala 1989). These findings may eventually bring studies on plant oscillations from a category of being merely "curious phenomena" to an important practical aspect of controlling plant growth and development.

18.4 Conclusions and Future Perspectives

It is obvious that ultradian oscillations in plants are more widespread than might appear at first glance. Being governed by non-linear mechanisms, virtually every physiological parameter in a plant may (and *should*) display oscillatory behaviour under certain conditions. The evidence for important physiological roles of such oscillations is beginning to emerge. Future research should be focused on molecular and cellular aspects of mechanisms, underlying such oscillatory behaviour at various levels of plant structural organisation, as well as on more direct evidence for the physiological role of oscillations in plant growth, development and adaptive responses to the environment. As for now, despite the phenomena being known for hundreds of years, we have only examined the tip of the iceberg.

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19 Electrical Signals in Long-Distance Communication in Plants

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Abstract Action potentials (APs) belong to long-distance signals in plants. They fulfill the all-or-none law, propagate without decrement and their generation is limited by refractory periods. The ion mechanism of APs was elaborated in giant *Characean* algae and extended by another model plant – the liverwort *Conocephalum conicum*. It consists of an increase in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) which activates anion channels responsible for Cl^- efflux and for membrane depolarization. Repolarization occurs after the opening of potassium channels and K^+ efflux. The resting potential is restored by the electrogenic proton pump. A number of ion channels which may play a role in AP were identified by the patch-clamp technique. APs propagate on the principle of local electrical circuits. They cover whole plants, plant organs or definite tissues, mainly phloem, phloem parenchyma and protoxylem. APs mediate between local stimulation and movements in carnivorous *Dionaea muscipula*, *Aldrovanda vesiculosa*, and tigmonastic *Mimosa pudica*. The role of APs in regulation of respiration, photosynthesis, growth, pollination, fertilization and gene expression is well documented. An AP-coupled increase in $[Ca^{2+}]_{cyt}$ seems to play a central role in signal transduction.

19.1 Action Potentials

19.1.1 General Characteristics

Action potentials (APs) are widespread signaling phenomena, known mainly from excitable animal tissues comprising nerves, muscles and epithelia. They exist in plants and fungi as well (Pickard 1973; Davies 1987). There are several criteria that allow APs to be distinguished from other phenomena having electrical components.

- APs can propagate with fairly constant velocity and without decrement.
- They are of all-or-none character, i.e., stimuli weaker than a certain threshold do not evoke APs, whereas overthreshold stimuli trigger APs of constant amplitude.
- Refractory periods: absolute and relative follow AP generation.

APs are also characterized by a regular time course, in contrast to wound-induced variation potentials (VPs) (Fig. 19.1). In APs the fast depolarizing

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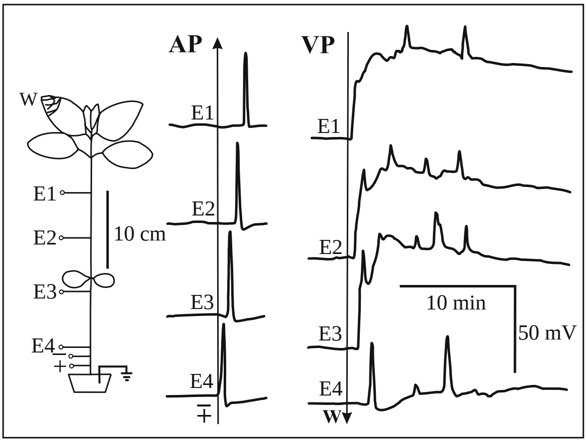

Fig. 19.1. Action potentials (*APs*) and variation potentials (*VPs*) recorded in the stem of *Helianthus annuus* by extracellular electrodes, *E1–E4*. The AP was elicited by electrical stimulation (±), and the VP by wounding (*W*). *Vertical arrows* indicate the moment of stimulation. *Arrowheads* point to the direction of propagation (After Stankovic et al. 1998)

phase is followed by slower repolarizing and after-hyperpolarizing phases. APs can be evoked by relatively weak, nondamaging stimuli. The same place can be stimulated many times without visible damage. APs spread on the basis of local electrical circuits.

19.1.2 Ion Mechanism of Action Potentials

The ion mechanism of APs was intensely studied in giant *Characean* cells. The huge dimensions of internodal cells enable application of many experimental techniques, including voltage-clamp, patch-clamp with a patch-pipette attached to the plasma membrane through a "window" cut in a cell wall, and calcium imaging with aequorin or fura microinjected into a cell. The cells can be surgically modified by cutting off the nodes and subsequent exchange of the vacuolar sap and even tonoplast decomposition (Tazawa and Shimmen 1987).

In resting cells Ca^{2+} and Cl^- are kept far from the electrochemical equilibrium. These two ion species are the best candidates as depolarizing ions. Indeed, according to the generally accepted model, an AP is initiated by calcium influx into the cytosol followed by Cl^- efflux (Williamson and Ashley 1982; Lunevsky et al. 1983). Chloride ions leave the cell down their electrochemical potential gradient through Ca^{2+}-activated anion channels. Repolarization occurs after opening of voltage-gated potassium channels of

K_{out} type allowing K^+ efflux. Chloride and potassium fluxes in *Chara* during one AP are much higher than the theoretical value of 1–2 pmol cm^{-2} and reach up to 10,000 pmol cm^{-2} (Oda 1976).

Recently, the question arose of what is the source of Ca^{2+} ions entering the cytosol (Tazawa and Kikuyama 2003). There is strong evidence that Ca^{2+} influx originates from the apoplast. Internal stores, mainly endoplasmic reticulum (ER), are indicated as an alternative source of Ca^{2+} ions. The former conclusion is supported, among others, by experiments with $^{45}Ca^{2+}$ and near-Nernstian dependence of the AP amplitude on external Ca^{2+} concentration (Hayama et al. 1979). The latter statement is based mostly on evidence that showed (1) a reduction of Ca^{2+} currents after application of IP_3 modulating factors (Biskup et al. 1999) and (2) quenching of fura fluorescence in cells preincubated with Mn^{2+} which coaccumulates with Ca^{2+} in the ER (Plieth et al. 1998). Combining these two sources of Ca^{2+} is also possible in a process of calcium-induced calcium release. According to it, a small portion of Ca^{2+} crossing the plasma membrane from the apoplast would lead to Ca^{2+} release from the ER and possibly other internal stores, including the vacuole. An increase in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is postulated to mediate between the AP on the plasmalemma and that on the tonoplast. The regulation of transient $[Ca^{2+}]_{cyt}$ increase during the AP is very precise and occurs according to the all-or-none rule (Wacke and Thiel 2001). Involvement of voltage-dependent IP_3 production in the mechanism of the AP is postulated (Wacke et al. 2003).

There were numerous attempts to characterize ion channels carrying individual ion fluxes coupled with the AP, using the patch-clamp technique. At least three calcium-dependent anion channels have been characterized in the *Chara* plasmalemma. Channels of unitary conductance of 9 pS (Okihara et al. 1991), 17 and 38 pS (Homann and Thiel 1994) have been examined. The 9-pS channel has a narrow $[Ca^{2+}]_{cyt}$ dependence with a maximum at approximately 1 μM. Addition of calmodulin transiently activates the channel, while calmodulin inhibitors W-7 and chlorpromazine cause its blockage (Okihara et al. 1993). The other two channel types show bursting behavior in a cell-attached mode. Quantal release of Ca^{2+} from stores in the vicinity of the plasmalemma was postulated to activate these channels (Thiel et al. 1993; Thiel and Dityatev 1998).

A possible role in conducting an outward current in the repolarization phase of the AP was attributed to the 40 pS potassium channel in *Chara* (Homann and Thiel 1994). In *Nitellopsis* a 25–50 pS K^+ channel was found (Katsuhara et al. 1990). The channel is regulated by external Ca^{2+} concentration and by ATP. Other nucleotides like AMP and the nonhydrolyzable ATP analogue are equally active in decreasing the channel open probability (Katsuhara and Tazawa 1992).

The picture is not so clear when calcium channels are considered. Thiel et al. (1993) found 4 pS Ca^{2+}-permeable channels in the *Chara* plasmalemma. It is postulated that they serve in Ca^{2+} accumulation in the vicinity of the plasmalemma. The channels do not show clear voltage dependence.

There is a general problem with application of the patch-clamp technique to study the channels operating during the AP. In most cases protoplasts are unexcitable. Thus, voltage-clamp experiments on intact turgid cells seem more reliable. The early studies by Lunevsky et al. (1983) and subsequent studies by Tsutsui and Ohkawa (1993) allowed the Ca^{2+} currents to be characterized in more detail. The pharmacology of channels carrying Ca^{2+} resembles that of L-type calcium channels in animal cells.

Characean cells offer technical advantages over small cells of higher plants, but because of their enormous dimensions they are highly specialized. Thus, their usefulness as model cells in explaining excitation in plants is often questionable. *Conocephalum conicum* – a liverwort – seems a better model system for studying APs in terrestrial higher plants. *C. conicum* belongs to the evolutionarily oldest land plants. The thallus has a simple structure with relatively large cells connected symplasmically. It has no conducting bundles. All cells including rhizoids are excitable. APs can be evoked, among others, by electrical stimulation, illumination and cooling. Wounding often leads to the generation of long-lasting trains of APs (Paszewski et al. 1982). APs fulfill all basic electrophysiological principles. They are generated according to the all-or-none law and they propagate throughout the thallus with a velocity of 2.5–9 cm min^{-1}. Immersion, depending on the resistance of the solution, causes acceleration of propagation to more than 30 cm min^{-1} (Zawadzki and Trebacz 1985). Excitation is always followed by refractory periods: absolute 2–4 min and relative 6–8 min (Dziubinska et al. 1983). Spatial and temporal summation of subthreshold stimuli takes place (Trebacz and Zawadzki 1985). *C. conicum* can be excited by light stimuli. When shaded, its thallus cells hyperpolarize and depolarize upon reillumination in a dose-dependent manner. Light-induced generator potentials, when strong enough, lead to exceeding of a threshold and AP generation (Trebacz and Zawadzki 1985).

Intracellular microelectrodes combined with application of ion channel and proton pump inhibitors allowed qualitative determination of ions participating in resting potentials and APs. In *C. conicum* the resting potential consists of passive and active components. The first is connected with the plasma membrane permeability to potassium and the other with operating of the electrogenic proton pump. Abolishing the active component by proton pump inhibitors causes depolarization accompanied by a decaying series of APs after which the cells became unexcitable. Treatment of *C. conicum* cells with TEA, which blocks K^+ channels, makes them nonresponsive to electrical stimulation. AP reduction or complete blockage was

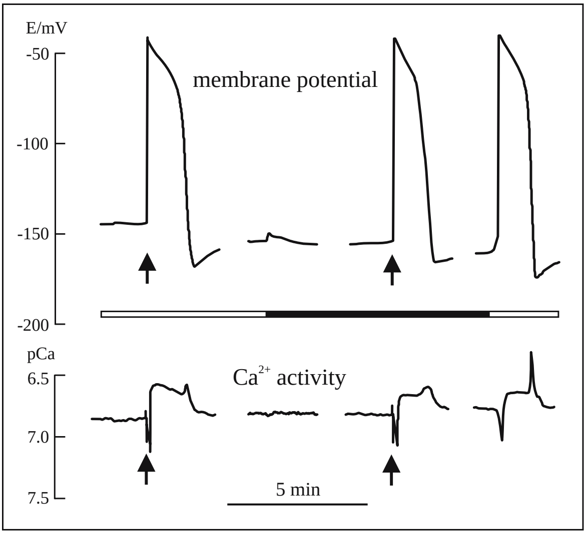

Fig. 19.2. Membrane potential changes (*upper traces*) and cytoplasmic Ca^{2+} activity (*lower traces*) measured in *Conocephalum conicum* by ordinary and ion-selective microelectrodes, respectively. *Arrows* indicate electrical stimulation. *White* and *black bars* denote light and darkness, respectively (After Trebacz et al. 1994)

observed after application of Ca^{2+} or anion channel inhibitors (Trebacz et al. 1989). These findings were confirmed after employing ion-selective microelectrodes. It was found that during the AP $[Ca^{2+}]_{cyt}$ increases from resting 231 to 477 nM (Fig. 19.2) while Cl^- and K^+ activities in the cytosol decrease (Trebacz et al. 1994).

Application of A-9-C, an anion channel inhibitor, together with tetraethylammonium (TEA) discloses voltage transients (VTs) evoked by light or cold stimuli, which no longer have all-or-none character (Fig. 19.3). Their amplitude depends on the stimulus strength and the period preceding stimulation. There is evidence that VTs represent a calcium component of APs (Trebacz et al. 1997; Krol and Trebacz 1999; Krol at al. 2003). In the absence of A-9-C and TEA, the VT is short-circuited by a high membrane conductance caused by the opening of anion and potassium channels during the AP. A VT is a suitable tool to investigate the sources of $[Ca^{2+}]_{cyt}$ increase. Reduction of the VT amplitude by impermeable lanthanides and its dependence on external Ca^{2+} concentration points to the apoplast as a source of Ca^{2+}. On the other hand, suppression of the VT by neomycin, an inhibitor of phospholipase C catalyzing IP_3 production, and magnification of the VT by Sr^{2+}, which is known to liberate Ca^{2+} from internal stores, is evidence that those Ca^{2+} sources play a role in the ion mechanism of the AP (Krol et al. 2003).

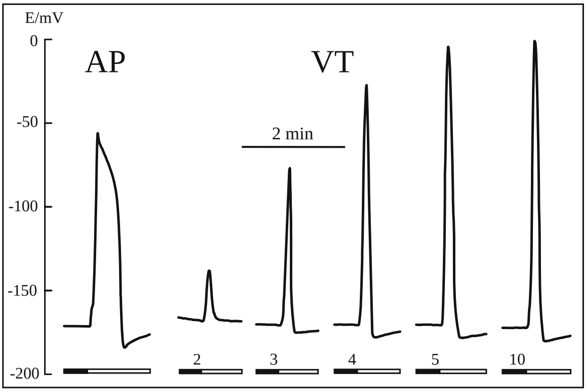

Fig. 19.3. AP and voltage transients (*VTs*) elicited by light in *C. conicum*. *Numbers* over *black bars* denote the duration of darkness period preceding illumination (After Trebacz et al. 1997)

Different aspects of ion mechanism of APs were also studied in higher plants. Special attention was paid to carnivorous plants *Dionaea muscipula* and *Aldrovanda vesiculosa* as well as seismonastic *Mimosa pudica*. In *Aldrovanda* Ca^{2+} influx during the AP was estimated as 8 pmol cm^{-2} (Iijima and Sibaoka 1985), which points to calcium influx as a trigger of subsequent ion fluxes. Potassium efflux was much higher – 6,200 pmol cm^{-2}. It was suggested that a charge balancing current was carried by Cl^- (Iijima and Sibaoka 1983). The data concerning the ion basis of APs in *D. muscipula* are mostly indirect. The dependence of the AP amplitude on an external Ca^{2+} concentration and its reduction by La^{3+} and ethylene glycol bis(2-aminoethyl ether)tetraacetic acid points to a calcium influx (Hodick and Sievers 1988). Repetitive electrical stimulation or a blockage of Ca^{2+}-ATPase, both leading to accumulation of Ca^{2+} in the cytoplasm, causes gradual reduction of AP amplitudes (Trebacz et al. 1996). The chloride component previously questioned (Hodick and Sievers 1988) was recently demonstrated (Krol et al. 2005).

In a leaf pulvinus of *M. pudica* large asymmetric K^+ and Cl^- effluxes with higher amounts of the ions in the lower part were demonstrated (Samejima and Sibaoka 1982; Kumon and Suda 1984). Ion channels in protoplasts from *M. pudica* pulvini were examined by the patch-clamp technique (Stoeckel and Takeda 1993). Delayed outward-rectifying potassium channels were found. Some of the protoplasts showed an N-shaped *I*/*V* curve characteristic of excitable cells (Stoeckel and Takeda 1993).

The Ion mechanism of APs was also studied in other higher plants exhibiting no fast movements. Fromm and Spanswick (1993) confirmed participation of Ca^{2+}, Cl^- and K^+ fluxes during the AP in *Salix viminalis*.

A significant role of the proton pump in AP electrogenesis was demonstrated in *Cucurbita pepo* by Opritov et al. (2002). Recently Fisahn et al. (2004) provided evidence that the AP in *Solanaum tuberosum* is accompanied by a substantial increase in $[Ca^{2+}]_{cyt}$. The data suggest that ryanodyne-receptor-coupled internal stores release Ca^{2+} during the AP. In marine diatoms (*Odontella sinensis*) fast (milliseconds) Na^{+}-based APs, like in animal cells, were registered (Taylor and Brownlee 2004). The abundance of sodium in seawater might be a reason why such a scheme of excitation is frequent in marine plants.

Investigation of the detailed nature of APs in terrestrial higher plants is still necessary. Very little is known about the regulation of excitability. Characterization of ion channels on a molecular level would help us to understand such processes.

19.1.3
Ways of Action Potential Transmission

The problem of electrical signal transmission in plants has recently been discussed by Dziubinska (2003). APs spread within cells on the basis of local electrical circuits. The mechanism is the same as AP transmission along axons. Cylindrical cells of *Characean* algae are suitable objects to demonstrate the principles of local circuit and cable theories. Longitudinal phloem cells of higher plants can also serve as a good analogy of axons. In many plants, like *Aldrovanda*, *Dionaea* and *Conocephalum*, transmission of APs resembles epithelial transmission of excitation in animals (Mackie 2004). Cells connected with plasmodesmata constitute a network which is able to transmit APs in different directions. Plasmodesmata or sieve pores in phloem serve as low-resistance bridges allowing AP transmission from cell to cell. In higher plants phloem, phloem parenchyma or protoxylem were shown to be suitable for AP transmission. The AP may gradually cover all living tissues in the shoot (Zawadzki and Trebacz 1982; Dziubinska et al. 2001). APs are sometimes limited to certain organs or parts of plants. For instance, in *Dionaea* all cells in the leaf converted into a trap can generate APs (Hodick and Sievers 1988), whereas the remaining parts of the leaf are totally unexcitable. In *Mimosa* the AP travels along the leaf and the petiole to the main pulvinus, where it stops. In *Lupinus angustifolius* the AP is transmitted along the shoot, both acropetally and basipetally, but does not enter leaves and roots (Zawadzki 1980). There are reports showing spontaneous generation of APs (Zawadzki et al. 1995). Extracellular electrodes recorded APs of different amplitudes transmitted along limited distances. It was concluded that spontaneous APs can be transmitted only down the cell lines having low-resistance plasmodesmata connections.

19.1.4
Physiological Implication of Plant Excitation

APs, like other signals, besides input(s) and the ways of transmission have to posses output(s), i.e., their passage has to "inform" distant cells about locally acting stimuli and let them respond appropriately. Before beginning a study of the physiological implication of APs, it is necessary to elaborate detailed electrophysiological characteristics of the plant examined. One has to find out if the signal is a real AP, which stimuli can evoke the AP, and how often they can be applied. It is also important to know what the velocity of AP transmission is, and which plant organs and tissues are excitable. In experiments aiming at checking the consequences of APs it is also desirable to break the AP transmission between the site of stimulus application and AP destination.

Trap closures of carnivorous *D. muscipula* and *A. vesiculosa* are among the best-documented consequences of excitation in plants. In *Dionaea*, bending of one of the trigger hairs protruding from the upper part of the trap leads to generation of an AP which spreads over the trap with a velocity of approximately 10 cm s^{-1}. What is important is that the trap does not visibly move after the first stimulation. It is necessary that the second bending of any of the trigger hairs occurs no later than 40 s after the first, to make the trap close. The second stimulus is accompanied by the second AP, whose velocity is much higher than that of the first one (up 25 cm s^{-1}) (Sibaoka 1969). Such a double-excitation-triggered trap closure protects the plant against an accidental stimulation. Trap reopening is an energetically wasteful process. The response to the second AP can be regarded as plant memory. Following the second AP the leaf closes quickly. The closure is, however, not complete. Small pray can leave the trap. When the victim is too large to escape and strong enough to bend trigger hairs many times in its struggle, the trap closes tightly and digestive glands begin to release enzymes that decompose its body. In spite of more than 100 years of AP investigation in *Dionaea* a detailed mechanism of its trap closure is not known. Hodick and Sievers (1989) demonstrated that all cells within the trap are excitable. There is also no special motor zone. Such cells are separated neither anatomically nor electrophysiologically. There is a delicate balance between tissue tensions in the trap which is shifted towards the closure after the second AP. Trap movement consists of relatively slow followed by very fast phases. The other phase is attributed to mechanical properties of the trap (Forterre et al. 2005). The question is why only the second AP is able to release the tension. Probably the second AP, when repeated soon enough after the first one, causes sufficient accumulation of Ca^{2+} in the cytosol and the release of Cl^- and K^+ that changes turgor, cell wall extensibility or makes the cytoskeleton rebuild.

The coupling between APs and functioning of digestive glands remains elusive. The presence of the prey is not necessary to induce the process. It is possible to evoke an extensive release of a digestive fluid by electrical stimulation of an immobilized trap (Trebacz, unpublished).

A. vesiculosa, a close relative of *Dionaea*, shuts down its traps after a single stimulation of a trigger hair followed by generation of a single AP (Iijima and Sibaoka 1982). When the trap receives only one stimulus it begins to open after 1 h. More stimuli make that the trap lobes press tightly against each other. Thus, in *Aldrovanda*, the system protecting against accidental stimulation is somewhat simpler than that in *Dionaea*. In stagnant water, where it grows, such a sophisticated mechanism does not seem necessary. Iijima and Sibaoka (1983) pointed out that traps of *Aldravanda* possess a motor zone consisting of cells located in a central part of the trap between two epidermal layers. A massive efflux of ions attributed to that zone causes a loss of turgor and a sudden trap closure.

The physiological significance of AP-regulated movements of leaves and pinna-rachis in other plant species, like *M. pudica*, *Biophytum dendroides* and *Desmodium motorium* is a matter of discussion, although the coupling between the AP and the movement is well documented (Sibaoka 1973; Antkowiak and Engelmann 1995).

In *Mimosa*, it was shown that not only the movement but also a photoassimilate unloading from the phloem occurs following an AP (Fromm and Eschrich 1988). A similar effect was demonstrated in *Zea mays*, which makes it probable that it is common in excitable plants.

APs also play a role in plant pollination and fertilization. The most spectacular example was described by Sinyukhin and Britikov (1967) in *Incarvillea*. Its flowers possess a bilobal stigma which closes upon mechanical stimulation exerted by a falling pollen grain. The movement which is mediated by the AP leads to pollen grain arrest inside the stigma. Soon after that, the second AP travels to the ovary, where it evokes a significant increase in respiration long before fertilization. APs evoked by pollination were also found in *Lilium* and *Hibiscus* (Fromm et al. 1995). The large dimensions of the pistils, which made electrode attachment easy, determined the selection of these objects. It is possible that the phenomenon is more general in flowering plants. APs were registered during movement of stamens in *Berberis* and *Mahonia* too (Simons 1981). APs were postulated to mediate between illumination and guttation in gametophytes of the moss *Bryum pseudotriquetrum* (Sinyukhin 1973). Drops of liquid appearing by guttation enable fertilization.

Excitation also influences gas exchange in plants. The significant increase of respiration following stimulation of the *C. conicum* thallus is well documented (Dziubinska et al. 1989). It was demonstrated that both damaging (cutting of a thallus edge) and nondamaging electrical stimuli

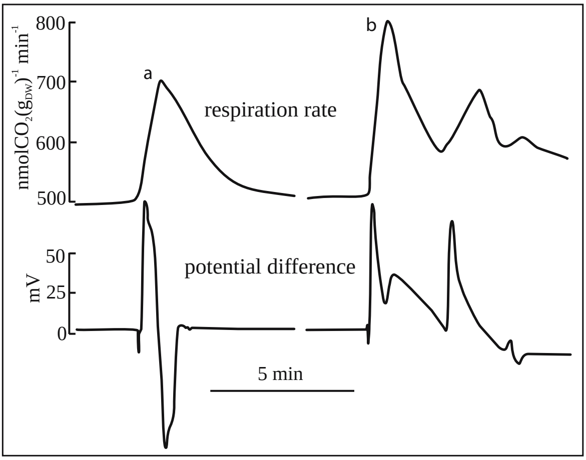

Fig. 19.4. Respiration rate (*upper traces*) and APs (*lower traces*) in *C. conicum*. **a** Response to a single electrical stimulus; **b** response to cutting a thallus edge (After Dziubinska et al. 1989)

evoked a burst of respiration provided an AP had been generated. In the thalli preincubated with TEA, in which excitability was blocked, no significant change in the respiration rate occurred in spite of wounding. In untreated plants when cutting released a series of APs, each AP in the sequence was followed by enhancement of respiration (Fig. 19.4). Changes in respiration rate concomitant with AP generation were registered among others in *Cucurbita pepo* and *Vicia faba* (Gunar and Sinyukhin 1963; Filek and Koscielniak 1997). There are also reports on changes in the level of photosynthesis evoked by stimuli able to evoke APs (Fromm and Eschrich 1993; Koziolek et al. 2003). The list of remaining physiological consequences of plant excitation covers, among others, temporary lowering of the growth rate in *Luffa cylindrica* (Shiina and Tazawa 1986) and *Helianthus annuus* (Stankovic et al. 1998), a decrease in susceptibility to cold stress in *Cucurbita pepo* (Retivin et al. 1997), enhancement of peroxidase activity in *Conocephalum conicum* (Dziubinska et al. 1999) and induction of jasmonic acid biosynthesis in *Solanum tuberosum* (Fisahn et al. 2004).

APs are also found to play a role in gene expression. Special attention was paid to regulation of the PINII gene whose products play a key role in a systemic response to wounding (Wildon et al. 1992). There was controversy as to the nature of the electrical signal preceding the response. Stankovic and Davies (1996) demonstrated that both APs and VPs induce PINII gene expression.

A frequently asked question is: how can such a uniform signal as an AP evoke such different responses or, more precisely, how can ion fluxes and

membrane potential changes alter enzyme activities and even play the role of transcription factors? Calcium seems a universal messenger or trigger in most of these processes. The network of mechanisms controlled by Ca^{2+} is very large (Hetherington and Brownlee 2004). The AP evolved as a factor spreading a calcium signal. The kind of response and its degree depend on the susceptibility of individual cells or tissues to Ca^{2+} fluxes. The concepts of "calcium signature" and "physiological address" seem to apply to APs.

19.2 Conclusions and Future Perspectives

Electrical signals in plants have been registered since the second half of the nineteenth century. The number of excitable plant species recorded is increasing. Knowledge about AP mechanism and physiological consequences is accumulating but there is still a broad margin for questions and speculations. Among the questions that have to be addressed in future investigations are the following:

– Which ion channels participate in APs? What is their molecular identity?
– How are they regulated in the short term (activation/inactivation) and how are they modulated (long-term changes in their number and/or posttranslation modification)?
– What are the roles of the vacuole, the ER and other intracellular structures in electrogenesis of an AP?
– What is the mechanism of diurnal and seasonal changes in excitability?
– How do plants regulate AP transmission?
– What is the precise mechanism of electrical signal transduction, i.e., a coupling between ion fluxes and physiological responses?

Answering these questions will help us to consider electrical signals in plants as normal phenomena and not as atavisms or extraordinary processes in extraordinary plants known only to a narrow group of plant electrophysiologists.

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20 Slow Wave Potentials – a Propagating Electrical Signal Unique to Higher Plants

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Abstract Plants have at least three kinds of propagating electrical signals. In addition to a sustained wound potential (WP) that stops a few millimeters from dying cells, these signals are action potentials (APs) and slow wave potentials (SWPs). All three signals consist of a transient change in the membrane potential of plant cells (depolarization and subsequent repolarization), but only SWPs and APs make use of the vascular bundles to achieve a potentially systemic spread through the entire plant. The principal difference used to differentiate SWPs from APs is that SWPs show longer, delayed repolarizations. Unfortunately, SWP repolarizations also show a large range of variation that makes a distinction difficult. SWPs and APs do differ more clearly, however, in the causal factors stimulating their appearance, the ionic mechanisms of their depolarization and repolarization phases as well as the mechanisms and pathways of propagation. The depolarizations of a SWP arise with an increase in turgor pressure cells experience in the wake of a hydraulic pressure wave that spreads through the xylem conduits after rain, embolism, bending, local wounds, organ excision and local burning. The generation of APs occurs under different environmental and internal influences (e.g. touch, light changes, cold treatment, cell expansion) that – mediated through varying generator potentials – trigger a voltage-dependent depolarization spike in an all-or-nothing manner. While APs and WPs can be triggered in excised organs, SWPs depend on the pressure difference between the atmosphere and an intact plant interior. High humidity and prolonged darkness will also suppress SWP signaling. The ionic mechanism of the SWP is thought to involve a transient shutdown of a P-type H^+-ATPase in the plasma membrane and differs from the mechanism underlying APs. Another defining characteristic of SWPs is the hydraulic mode of propagation that enables them – but not APs – to pass through killed or poisoned areas. Unlike APs they can easily communicate between leaf and stem. SWPs can move in both directions of the plant axis, while their amplitudes show a decrement of about 2.5% cm^{-1} and move with speeds that can be slower than APs in darkness and faster in bright light. The SWPs move with a rapid pressure increase that establishes an axial pressure gradient in the xylem. This gradient translates distance (perhaps via changing kinetics in the rise of turgor pressure) into increasing lag phases for the pressure-induced depolarizations in the epidermis cells. Haberlandt (1890), after studying propagating responses in *Mimosa pudica*, suggested the existence of hydraulically propagated electric potentials at a time when only APs were conceivable. It took a century to realize that such signals do exist and that they coincide with the characteristics of SWPs rather than those of APs. Moreover, we begin to understand that SWPs are not only ubiquitous among higher plants but represent a unique, defining characteristic without parallels in lower plants or animals.

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20.1 A New Effort to Decipher the Impact of Electrical Long-Distance Signals in Plants

For a long time plants were thought to be organisms whose limited ability to move and respond was matched by limited abilities of sensing. Exceptions were plants with rapid, purposeful movements such as *Mimosa pudica*, *Droseras* (sundews), *Dionea muscipula* (flytraps) and tendrils of climbing plants. These "sensitive plants" attracted the attention of researchers like Pfeffer, Burdon-Sanderson (1873), Darwin, and Haberlandt (1890; 1914). They found that these plants use sensitive mechanoreceptors and action potentials (APs) that implemented these movements. Although hardly appreciated at that time, the discovery that normal plants such as pumpkins had propagating APs just as the sensitive plants (Gunar and Sinykhin 1962, 1963) was an important scientific landmark. First, it corrected the long-held belief that normal plants are less responsive than sensitive plants. Second, it led to a new, eagerly pursued belief that such widely distributed electric signals must carry messages with an importance that could exceed the induction of organ movements in animals and sensitive plants. In different laboratories around the world this anticipation became the driving force for a renewed quest to decipher the meaning of electrical plant signals. Considerable progress was made in linking electrical signals with respiration and photosynthesis (Gunar and Sinykhin 1963; Koziolek et al. 2003), pollination (Sinykhin and Britikov 1967; Spanjers 1981), phloem transport (Opritov 1978; Fromm and Bauer 1994), rapid deployment of plant defenses (Wildon et al. 1992; Malone et al. 1994; Alarcon and Malone 1995; Herde et al. 1995, 1996; Stankovic and Davies 1996, 1998).

However, with only a few scattered laboratories producing results, new data suffered an almost constant lack of confirmation by other laboratories. This slow progress is traceable in the case of the plant-wide or systemic induction of proteinase inhibitors in wounded tomato plants, which was discovered as early as 1972 (Green and Ryan 1972). Although an involvement of wound-induced electrical signals was immediately suspected and tested (Pickard 1973; Van Sambeek and Pickard 1976), it took 20–30 years before the relationship was independently confirmed (Wildon et al. 1992; Malone et al. 1994; Herde et al. 1995, 1996; Stankovic and Davies 1996, 1998).

20.2 Propagating Depolarization Signals in Plants

Three different types of propagating depolarizations in plants have been suggested to reflect three different types of signals: APs, slow wave potentials

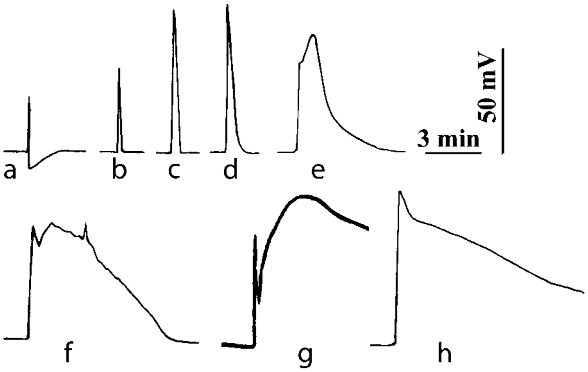

Fig. 20.1. Extracellular and intracellular recordings of the three major types of propagating depolarization signals (action potentials, *APs*, slow wave potentials, *SWPs*, and wound potentials, *WPs*). They show in *a* an AP recorded from the abaxial surface of a *Dionea muscipula* leaf after touching a trigger hair located on the opposite lobe at a distance of 15 mm, in *b* an AP recorded from the epicotyl of a 3-week-old, intact sunflower plant after reduction of bright illumination from 150 to 10 μmol m^{-2} s^{-1} white incandescent light, in *c* a SWP from the epicotyl of an intact sunflower plant after one puncture of the hypocotyl in air 17 cm below the recording site, in *d* a SWP from the epicotyl of an intact sunflower plant after one puncture of the hypocotyl under water and 20 cm below the recording site, in *e* a SWP from the epicotyl of an intact sunflower plant after a 2-mm-deep cut in the submersed surface of the hypocotyl 20 cm below the recording site, in *f* a SWP from the epicotyl of an intact sunflower plant after a 1-s-long torching of the tip of a canopy leaf 15 cm above the recording site, in *g* a combined signal of AP and SWP in the petiole of *Mimosa pudica* after burning of the leaflet tip (redrawn from Houwinck 1935; Sibaoka 1953; Roblin 1985), and in *h* a WP from the epicotyl of an intact sunflower plant after one puncture directly at the recording site. Although APs, SWPs and WPs differ in rates of repolarization, this difference can be too small for reliable identification

(SWPs, also called variation potentials) and wound potentials (WPs). All three signals represent a transient depolarization. However, only SWPs and APs use the vascular bundles to cover longer distances and potentially spread through the entire plant. Examples a and b in Fig. 20.1 show an AP from a flytrap leaf (a) that is of a very short duration and an AP from a sunflower stem (b) that – like most APs in higher plants – lasts for a much longer time of 30–50 s. Within this range APs are characterized by a rapid depolarization and a rapid repolarization. When the depolarization passes a certain threshold, excitable plant cells are able to amplify the signal to a full AP spike by strictly implementing the all-or-nothing rule. Therefore, within the same cell types, the propagation of such APs will proceed in the form of nondecrementing signals with constant amplitude.

SWPs were first discovered by recognition that their kinetic appearance was different from that of APs (Houwinck 1935; Sibaoka 1969, 1991; Umrath

1959). Electrical recordings in *Mimosa* showed an electrical excitation signal in the form of a sequential combination of a short AP spike and a longer wavelike SWP (Fig. 20.1, trace g). Such a sequence was understood to imply that the AP moved faster through the *Mimosa* leaf and therefore appeared earlier at the recording site at the petiole base than the slow wave. The same sequence of signals was found in *Vicia faba* (Roblin 1985), but in sunflowers and cucumbers SWPs can be faster or slower than APs (Stankovic et al. 1997; Stahlberg and Cosgrove 1997c).

Today the "slow" in SWP refers to the slow repolarization and the resulting wavelike appearance rather than to inferior propagation rates (Fig. 20.1, traces e–g). To date a low rate is the most frequently applied criterion to differentiate SWPs from APs (Stankovic et al. 1997; Dziubinska et al. 2001). However, even if measured in the same location (sunflower stem) SWPs can cover an astounding range in repolarization times and their resulting shapes closely approach that of either APs or WPs (Fig. 20.1, traces c–g). Repolarization times are extended after flame induction and root excision (Fig. 20.1, traces e and f), but short when initiated by a needle puncture (Fig. 20.1, traces c and d). SWP signals can be contaminated with action spikes (Fig. 20.1, traces f and g). Such mixed signals have been found among many species, e.g., tomatoes, cucumbers and sunflowers (Roblin 1985; Stahlberg and Cosgrove 1997c; Stankovic et al. 1998b). Moreover, uncontaminated SWPs occur in pea epicotyls (Stahlberg and Cosgrove 1992, 1994, 1995, 1996, 1997c) and perhaps *Tradescantia* shoots (Tsaplev and Zatsepina 1980). In addition to the slower repolarization, uncontaminated SWPs show slower depolarizations than APs and a round rather than pointed signal shape (see Fig. 20.1, traces e and g, plus SWPs from pea epicotyls in Stahlberg and Cosgrove 1992, 1996, 1997a). Unlike APs, SWP induction does not follow an all-or-nothing rule and SWP amplitudes therefore decrement during propagation (see later).

Finally, there are WPs as a direct depolarization response in the vicinity of injured cells (Fig. 20.1, trace h). WPs have very long repolarization times and show a range from less than 1-mm to 40-mm distance (Shimmen 2001; Stahlberg and Cosgrove 1994). The overlap in appearance of the depolarization–repolarization events makes it difficult to distinguish the three signals (Fig. 20.1); therefore, other distinguishing characteristics are needed, e.g., stimuli causing SWPs to appear, ionic mechanisms mediating depolarization and repolarization, in rates, mechanisms and pathways of SWP propagation. What then is a SWP and what are its characteristics?

20.3 SWPs are Hydraulically-Induced Depolarizations

The classic way to induce SWPs is to bring an open flame in contact with a leaf or another part of the plant (Houwinck 1935; Umrath 1959; Roblin 1985; Wildon et al. 1992; Stankovic et al. 1997; Dziubinska et al. 2001). Flaming was considered as a model wound stimulation and an entire set of indirect data suggested that the excitation in *Mimosa* and other plants was mediated by the transpirational transport of wound substances emanating from the burned site (Ricca 1916; Umrath 1959; Schildknecht 1984). However, heat increases gas volume and pressure in the intercellular spaces (reflected as an increase in leaf thickness; Malone 1992, 1996; Boari and Malone 1993) and – more importantly – transiently increases volume and pressure in the narrow xylem conduits of the vascular bundles (Stahlberg and Cosgrove 1997c). Therefore, flaming acts as a strong hydraulic signal that appears as a rapid increase in xylem pressure (Stahlberg and Cosgrove 1997c), turgor pressure (Malone and Stankovic 1991), growth rate (Stahlberg and Cosgrove 1992, 1996), and leaf and stem thickness (Boari and Malone 1993).

The idea that hydraulic signals are accompanied by an electrical depolarization was clearly expressed by two independent studies in the early 1990s (Malone and Stankovic 1991; Stahlberg and Cosgrove 1992). A hydraulically propagated signal had already been suggested to exist in flamed *Mimosa* leaflets (Haberlandt 1890) but experimental evidence for a hydraulic wave paralleling AP propagation did not materialize (Tinz-Fuchtmeyer and Gradmann 1990). It was 100 years later that the exposure of the root of intact pea seedlings to modest pressure steps showed the appearance and propagation of a well-resolved transient depolarization in the pea epicotyl (as in Fig. 20.6). This propagating electrical signal, however, was not an AP but had the typical shape and slow-repolarization characteristics of a SWP (Stahlberg and Cosgrove 1992, 1996, 1997a, 1997c).

The large, propagating depolarizations of a SWP are generated by the application of positive, not negative, steps in xylem pressure (Fig. 20.2b). Rapid axial propagation of the hydraulic signal is manifested by an almost immediate water uptake into the apical growth zone in both pea and sunflower shoots (Stahlberg and Cosgrove 1992, 1995, 1996; Stankovic et al. 1997) and equally rapid changes in turgor and xylem pressure (Malone and Stankovic 1991; Stahlberg and Cosgrove 1995). How does a pressure signal that is almost instantly rising throughout the stem axis relate to an electric signal that takes minutes to climb the stem? An analysis of this question in pea epicotyls found that the induced slow wave depolarizations increased amplitude, rate and range in proportion to the size of the applied pressure steps while their lag phases were declining (Fig. 20.3; Stahlberg and Cosgrove 1997a). Figure 20.3 explains an important point: depolarizations

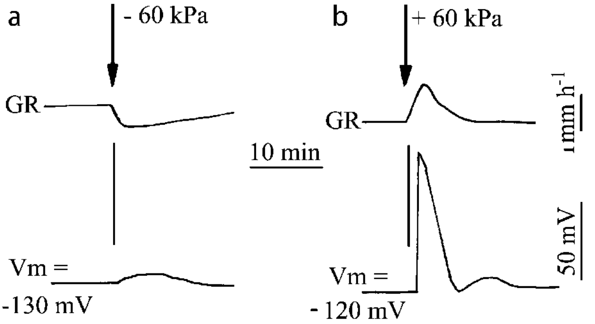

Fig. 20.2. The application of negative (vacuum) and positive pressure steps of equal size (60 kPa) to the submersed basal cut of an etiolated pea epicotyl shows that only positive pressure steps lead to increased growth rate (*GR*) and water uptake as well as the generation of a SWP depolarization, here measured at the epicotyl surface at a distance of 60 mm

will vary in amplitude and lag in appearance only if the xylem pressure in the measured stem section increases between 30 and 80 kPa. For pressure increases larger than 80 kPa, depolarizations will be identical, i.e., they will appear immediately and with similar, i.e., maximal, amplitudes. Thus, a SWP will appear immediately and simultaneously along entire stem or leaf sections as long as their change in xylem pressure exceeds 80 kPa (e.g., in monocot leaves after flame stimulation; Malone and Stankovic 1991; Malone 1992).

Unlike ideal tubes, xylem conduits leak water in a centrifugal direction and they do so preferably upon an increase in xylem pressure (Canny 1995). When a chamber enforced a constant pressure increase of 50 kPa to the vasculature of the submersed basal end of a pea stem segment, it was found this pressure is not transmitted in full amplitude to the apical end of the segment (Fig. 20.4). The transmitted pressure steps decline with increasing length of the measured segments and completely disappear when the epicotyl length exceeds 12 cm. While Fig. 20.4 shows a linear drop of the xylem pressure from the base to the tip of the pea epicotyl, Fig. 20.3 predicts that an axial pressure gradient in the range 30–80 kPa should create a series of depolarizations with increasing lag and decreasing amplitudes. Figures 20.3 and 20.4 therefore provide the basis for understanding SWPs. The apparent, decremented apical "movement" of a SWP is not due to genuine axial propagation but to delayed electrical responses to increasingly smaller hydraulic signals (Stahlberg and Cosgrove 1997c). One cannot avoid being impressed by the accuracy with which plant stems translate pressure steps into electrical signals, distances into increasing lags of the depolarization produced, and by the reliability with which these computations create an always perfect illusion of an electrical signal propagating along the surface.

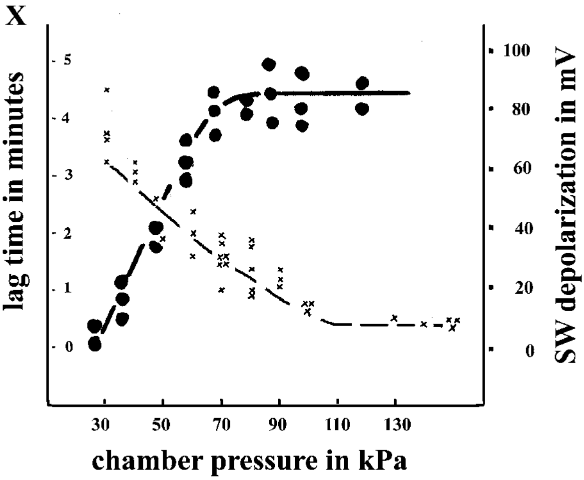

Fig. 20.3. A compilation of measurements shows that SWP characteristics such as lag phases (*crosses*) and amplitudes (*circles*) of SWP depolarizations depend on the size of the xylem pressure steps (which are somewhat smaller than the actually shown pressure steps that were applied to the submersed cut end of an etiolated pea seedling at a distance of 60 mm from the recording site, see Fig. 20.4). Note that a propagating SWP appears only at lower pressure steps. At pressures above 100 kPa the induced slow wave (*SW*) depolarizations become indistinguishable in time of appearance (lag phase→0 s) and amplitude. (Compiled from unpublished and published data from Stahlberg and Cosgrove 1997a)

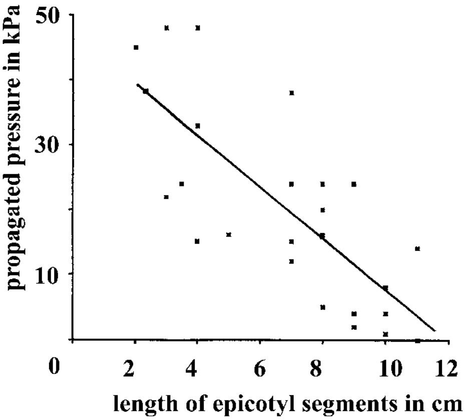

Fig. 20.4. Regression analysis of pressure propagation in epicotyl segments of various lengths shows the linear dissipation of a constant pressure step of 50 kPa from the basal end of application towards the apex. The basal ends of pea epicotyl segments 2–12 cm long were submersed and sealed into a pressure chamber while the apical end was sealed to a pressure probe. A loss of about 4 $kPacm^{-1}$ reflects the radial leakiness of the xylem. (Redrawn from Stahlberg and Cosgrove 1997a)

The SWP phenomenon suggests that the epidermal cells of many species and organs show a depolarization as a consequence of a rapidly rising turgor pressure. The quantitative characterization of this relationship, e.g., with the use of a combination probe measuring turgor pressure and membrane potential, would fill the remaining void about the hydraulic induction of the SWP. Pressure steps can become severely reduced on their centrifugal path from the xylem to the epidermis (Westgate and Steudle 1985). Application of 100 kPa pressure steps to 3 cm-long epicotyl segments was insufficient to cause a measurable increase in epidermal turgor pressure (Stahlberg and Cosgrove 1992). Stankovic and Malone (1991) measured large turgor pressure changes in the epidermal cells of torched wheat leaves but not the increase in xylem pressure. Together with the radial dissipation of xylem pressure steps, a parallel study of the radial propagation of the depolarization from the vascular bundles to the epidermis would be useful to fully understand the conversion of pressure into electrical signals. So far such studies exist only for APs (Rhodes et al. 1996; Herde et al. 1998).

Induction of SWPs by small pressure steps applied without injury to intact plants also presents a powerful argument against participation of chemical wound factors and for a purely hydraulic induction of SWPs (Stahlberg and Cosgrove 1995, 1996). In spite of this, it is still considered that some plant species may use electrogenic substances to induce propagating electrical signals. The idea draws support from the finding that raw extracts from *Mimosa*, *Biophytum* and tomato plants were able to induce propagating depolarizations (Ricca 1916; Umrath 1959; Van Sambeek et al. 1976; Cheeseman and Pickard 1977; Sibaoka 1997). On the other hand, feeding of wound sap to excised pea epicotyls showed clearly that peas do not use wound substances for SWP generation (Stahlberg and Cosgrove 1992). Figure 20.5 shows a test of whether the xylem-mediated transport of strongly depolarizing agents like cyanide and azide is capable of generating a propagating depolarization in excised sunflower shoots. The induced signal moves slowly (less than 1 mm s^{-1}) in comparison with a hydraulically induced SWP (5–10 mm s^{-1}; e.g., Fig. 20.6) with depolarizations being sustained rather than transient. In order to produce SWP-like signals, potential excitation substances must (1) be shown to accumulate in sufficient quantity to cause a large and rapid depolarization, (2) be able easily to access and exit the vascular bundles and (3) cause transient depolarizations. None of the many SWPs recorded so far have been shown to fulfill these criteria.

While experimental methods of induction explore signal character and effects, it is equally important to find natural circumstances under which plants generate SWPs. Such situations include puncture wounds by sap-sucking insects (Alarcon and Malone 1994; Volkov and Haak 1995; Fig. 20.6); embolisms (Stahlberg and Cosgrove 1996), soil hydration during rains and

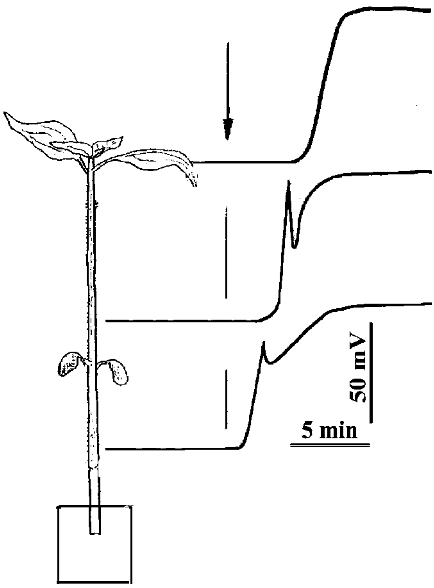

Fig. 20.5. After the submersed excision of the lower hypocotyl of an intact sunflower plant and a waiting period of 60 min that allowed the ascending transpiration stream to return to its normal rate, the basal end of the shoot was subjected to a 5 mM solution of sodium azide (*arrow*), an electrogenic model substance known to cause a large depolarization in plant cells. The transport and release of this substance from the xylem causes a wave of depolarizations that ascends the stem from the hypocotyl (*lower trace*; the distance to the basal cut was adjusted to be 10 cm) to the epicotyl (*center trace*; the distance to the basal cut was 20 cm) to a leaf blade (*upper trace*; the distance to the basal cut was 30 cm). The transport of this depolarizing chemical produces a signal that differs from a hydraulic SWP by a much lower propagation speed and the absence of repolarization and transience

floods (Stahlberg et al. 2005a), and perhaps also the reestablishment of positive xylem pressure during the night in root-pressure-generating plants, and strong bending of plants under wind and other mechanical influences.

For as long as they have been recognized as different entities, SWPs and APs have been believed to originate from different causes (Sibaoka 1953; Umrath 1959). While the cooling of roots and the application of small electric currents in the tissue seem to induce exclusively APs, the induction by heat (leaf flaming) has been reported to induce SWPs as well as APs (Roblin 1985; Wildon et al. 1992; Stankovic et al. 1997, 1998). Repeated flaming of sunflower leaves changed the shape of the resulting stem response from a clear SWP to an AP-like signal (Davies et al. 1991). These data indicate the possibility of an interaction between SWPs and APs that has not been investigated. Both APs and SWPs are propagated within the vascular

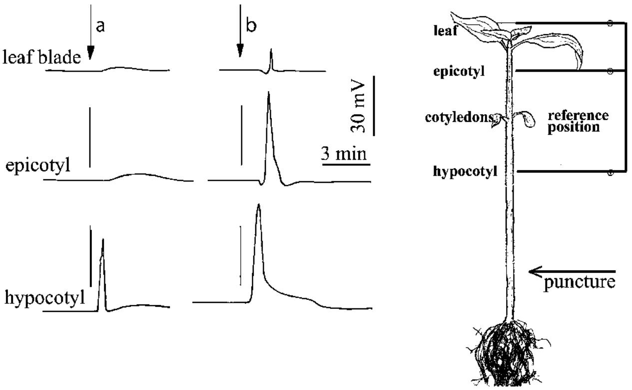

Fig. 20.6. SWPs in the stem of intact sunflower plants arise after a puncturing wound in either a nonsubmersed (**a**) or a submersed (**b**) part of the hypocotyl with a 0.4-mm-wide needle. Caused by the difference between external atmospheric pressure and a transpiration-dependent tension inside the plant, a pressure wave ascends from the wound up the stem in the punctured vascular bundle(s) with high speed and is followed by the appearance of a slower moving wave of depolarizations at the stem surface. In the case of a limited water supply at the puncture wound, the hydraulic signal exhausts itself and the range of the SWP ends between the first and the second electrode position at the stem (**a**). When the puncture is made in the submersed hypocotyl, the SWP becomes systemic, i.e., it increases its range to include all electrode positions along the stem (**b**). The data show that the range of wound-induced SWP depends on the availability of apoplastic water near the wound side

Table 20.1. Characteristics of slow wave potentials in comparison with action potentials and wound potentials. For details and references about the statements see the text

Characteristics of	Slow wave potentials	Action potentials	Wound potentials
Induction	Increase in turgor and xylem pressure	Depolarization beyond certain threshold	Turgor loss of neighboring cells
Amplitude	Graded; amplitude function of stimulus size	Amplitude fixed by all-or-nothing principle	Graded; depends on degree of injury
Induction methods	Heat, wounding, pressure chamber	Heat, cold, touch	Wounding
Ionic mechanism	P-type H^+ pump, ± ion channels?	Ca^{2+}, Cl^{2-} ion channels	P-type H^+ pump, ±ion channels?
Repolarization	Slow; > 1–30 min	Quick; < 1 min	Slow; 3–90 min
Propagation	As pressure signal in vascular bundles/xylem	As electric signal in vascular bundles/phloem	Unknown

bundles (Table 20.1). Since the depolarization of a SWP lingers longer than that of an AP, SWPs may be more effective than APs in triggering the opening of excitable, i.e., voltage-gated Ca^{2+} and anion channels needed for AP induction and propagation. SWPs in *Vicia*, cucumber and sunflower plants are frequently accompanied by action spikes (Roblin 1985; Roblin and Bonnemain 1985; Stahlberg and Cosgrove 1994, 1997a; Stankovic and Davies 1998; Stankovic et al. 1997). Conversely, the fact that no depolarization has ever been reported to cause a propagating SWP suggests that APs are unable to trigger SWPs.

20.4 The Propagation of SWPs

According to previously introduced considerations (Figs. 20.3 and 20.4), SWPs only undergo an apparent propagation with an appearance and range that is determined by the gradual decline of the inducing pressure signal. Unlike flames and pressure chambers, wounding by puncturing, surface cuts and organ excision generates a hydraulic signal that is based on existing pressure gradients in the plants (Stahlberg and Cosgrove 1997c). This provides useful information of how, e.g., species, age, light and other environmental conditions affect the ability of plants to generate SWPs as well as their size and range (Alarcon and Malone 1994; Stahlberg and Cosgrove 1994, 1995; Stahlberg et al. 2005). An example is given in Fig. 20.6.

Figure 20.6, example B shows the rapid ascent of a SWP from a puncture wound in the hypocotyl to the upper hypocotyl, epicotyl and leaf in an intact, illuminated sunflower plant. When the nonsubmersed part of a hypocotyl was punctured, the limited water supply near the punctured vascular bundle supported only a SWP with a short range limited to the hypocotyl (Fig. 20.6, example A). When punctured under water, the generated SWP had an increased, systemic range. The increased range is a clear indication of the hydraulic nature of SWP propagation. Using a stimulus that is close to the small scale of insect-inflicted wounds, we present here a short SWP that could be more representative than those published before as a consequence of larger injuries. It propagates with the same rate as those induced by root excision and shows the same range extension to wounding under water (Stahlberg et al. 2005b).

Although propagation rates of SWPs and APs overlap (Roblin 1985; Roblin and Bonnemain 1985; Stankovic et al. 1997), SWPs have four distinctive features that clearly separate their movement from that of APs. First, SWPs propagate with a measurable decrement (loss of amplitude) along the plant surface. Excision-induced SWPs in sunflower stems have a decrement of 2.5% cm^{-1} (Stahlberg et al. 2005b). Second, SWPs propagate from the stem

into the leaves and vice versa, whereas APs (at least in sunflowers) cannot do so (Dziubinska et al. 2001; Stahlberg et al. 2005b). A third defining particularity of hydraulic propagation is that it enables SWPs – but not APs – to pass through stem sections where the living cells were killed or poisoned (Stahlberg and Cosgrove 1992). Fourth, SWPs depend on the pressure gradient between the atmosphere and an intact plant interior being under tension. Means to specifically suppress SWP propagation include working with excised sections or intact plants under water or an atmosphere of 100% humidity (Mancuso 1998; Stahlberg et al. 2005b).

20.5 The Ionic Mechanism of SWPs

A highly negative membrane potential in plant cells characterizes an active state that involves the participation of the P-type H^+ pump and generates large potential gradients for ions such H^+, K^+, Ca^{2+} and Cl^- across the plasma membrane. Three major ion fluxes can generate large depolarizations in such cells: (1) an increased inward flow of Ca^{2+} ions from the cell walls into the cytoplasm, (2) an increased outward flow of Cl^- and other anions and (3) a rapid stop in the outward pumping of H^+ ions by the P-type ATPase.

While plant APs have been shown to involve the opening of ion channels (Sibaoka 1969, 1991; Fromm and Bauer 1994); SWPs are thought to reflect a transient shutdown of the P-type proton pump at the plasma membrane (Table 20.1). Evidence for this mechanism is that (1) SWPs depolarize cells by a maximal amount of 90–100 mV, leaving the membrane potential at a negative voltage near the Nernst potential for K^+ ions, (2) amplitudes of the SWP depolarization change continuously with the applied pressure size, (3) SW depolarizations are reduced or suppressed by the use of metabolic inhibitors (Julien et al. 1991; Stahlberg and Cosgrove 1992), (4) no measurable change in the cell-input resistance accompanied the large SWP depolarization of pea epicotyl cells (Stahlberg and Cosgrove 1992, 1996, 1997c), (5) SWP and the pH increase in the apoplast showed matching kinetics when measured with the fluorescent indicator DM-NERF (Stahlberg and Cosgrove 1996), and that the growth rate of apical stems drops with the arrival of the SWP signal (Stahlberg and Cosgrove 1997c; Stankovic et al. 1998). Being the fastest effect on this mechanism known (see Palmgren 1998) the hydraulic or stretch-activated inhibition of the P-type H^+ pump deserves more investigation. One cannot yet exclude that the ionic mechanism of SWPs could be more complex and in some species involve the participation of turgor-controlled or stretch-activated/inactivated ion channels. Unlike pea epicotyls, epidermal cells of cucumber hypocotyls show

transient increases in the cell input resistance during pressure-induced SWPs (Stahlberg and Cosgrove 1997c).

Why is the repolarization of SWPs slower than in APs? Repolarization of plant APs is believed to involve the combined action of voltage-dependent closures of the depolarizing ion channels, the voltage-dependent activation of repolarizing K^+ currents and an increased activity of P-type H^+ pumps. Never going far beyond the Nernst potential for K^+ ions, SWP depolarizations are not likely to be compensated by large outward K^+ currents. Another cause for the delayed repolarization of SWPs is the elimination of the role P-type H^+ pumps play in the repolarization efforts of plant cells. If the second stage of AP repolarization is mediated by a P-type H^+ pumping ATPase (as suggested by Orpitov et al. 2002 for cucurbit cells) a turgor-inhibited pump would explain the slower repolarization of SWPs.

SWPs share one important feature with APs and WPs; a refractory period during which the plant cells are unable to repeat the voltage signal when subjected to the same stimulus (Zawadzki et al. 1991). When a sequence of pressure steps was applied with 10 min intervals in between, all steps caused a transient increase in growth rate but only the first pressure application generated a SWP (Stahlberg and Cosgrove 1996). Systematic studies of refractory periods of SWPs in green plants are completely wanting.

20.6 The Effects of SWPs: Targeted Organs

SWPs trail hydraulic signals and very few studies differentiate whether an effect is caused by either the hydraulic or the electric component. One attempt was the comparison of the growth behavior of cucumber and pea seedlings before, during and after the passage of the electrical SWP signal into the growth zone (Stahlberg and Cosgrove 1997c). Application of small, sustained pressure steps to the stem base rapidly and transiently increased the growth rate due to a hydraulically mediated increase in apoplastic (and turgor) pressure. The delayed appearance of the electrical signal in the apical growth zone coincided with an unexpected, drastic drop in growth rate. The sustained slow wave depolarization in cucumbers paralleled a sustained growth inhibition of their hypocotyls, while a transient slow wave depolarization in peas had a transient effect on the epicotyl growth. Related results from sunflowers show a sustained shrinking of the upper stem after the passage of a flame-stimulated SWP (Stankovic et al. 1998).

Mobility of SWPs in both directions of the plant axis suggests two potential targets: the growing shoots with young canopy leaves and the root. In addition to stem growth, dramatic responses have been reported for leaves known to undergo particularly large, amplified SW depolarizations

(Stahlberg et al. 2005b). Leaf effects range from shutdown of stomata (Wildon et al. 1993; Pena-Cortes et al. 1995), to shutdown of photosynthesis (Koziolek et al. 2003), increased production of jasmonic acid and up-regulated transcription of proteinase inhibitor II and calmodulin in tomato plants (Herde et al. 1996; Stankovic and Davies 1998). Less is known about root responses to SWPs. Sunflower and cucumber plants develop root pressure that is metabolically supported and sensitive to pressure signals that can eliminate it in a rapid and drastic all-or-nothing manner (Stahlberg and Cosgrove 1997b). Leaf flaming generates basipetal electrical and pressure signals that could switch the root pump off and could cause in this way the observed flame-induced shrinking of sunflower stems (Stankovic et al. 1997). This has not been tested yet. Although there is almost no information on SWPs in roots, early work in *Vicia* indicates that the protein metabolism in roots is as sensitive to hydraulic signals as in shoots (Theilet et al. 1982).

20.7 WPs and SWPs

Although both APs and SWPs have been called wound signals, there is an electrical signal more deserving of this name. WPs occur not only in higher plants, but also in excised plant organs, nonvascular plants and algae (Shimmen 2001). A tandem pair of *Chara* internodal cells is a simple system to study cellular interactions. When one cell is damaged or killed, the neighboring cell undergoes a WP in the form of a large transient depolarization, sometimes with and sometimes without the occurrence of spikes (Shimmen 2001). Although cellular networks are more complex, cucumber hypocotyls showed identical responses (Stahlberg and Cosgrove 1994). WPs in cucumber hypocotyls extend for a distance of 40 mm (a length corresponding to about 200 epidermal cells), 10 mm in pea epicotyls (Stahlberg and Cosgrove 1994), 5 mm in corn roots (Chastain and Hanson 1982), and 1 mm in barley roots (Mertz and Higinbotham 1976). WPs appear as universal signals with specific extensions for different species and organs. Touch causes a similar response; a slowly repolarizing local depolarization with amplitudes that depend on the strength of the mechanical stimulus (Okamoto 1955; Zerrenthin and Stahlberg 1982).

Evidence exists from *Chara* tandem cells, sugar beet roots and cucumber hypocotyls that the size of the turgor pressure of the victim cell(s) before injury affects the amplitude of the generated WPs (Kinraide and Wyse 1986; Shimmen 2001; Stahlberg and Cosgrove 1997a). WPs in higher plants seem to be caused by a rapid inhibition of P-type H^+ pumps in the effected cells (Chastain and Hanson 1982; Gronewald and Hanson 1980; Kinraide

and Wyse 1986). Accordingly, WPs (1) are accompanied by a strong reduction in the growth rate of the cucumber hypocotyl in a 40-mm range from the wound site and (2) proceed without a change in cell input resistance (Stahlberg and Cosgrove 1994, 1997a). Although WPs and SWPs seem to share a similar ionic mechanism, WPs lack an important defining characteristic of SWPs: distant propagation (Table 20.1). SWPs may have evolved as a type of propagating WP.

Haberlandt (1890) suggested the existence of hydraulically propagated electric potentials at a time when the only known electrical signals were APs. It took time to find such signals and to understand that they coincide with SWPs rather than APs. We slowly begin to realize that SWPs are not simply ubiquitous but characteristic, defining signals for higher plants that are missing in lower plants or animals.

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21 Electrical Signals, the Cytoskeleton, and Gene Expression: a Hypothesis on the Coherence of the Cellular Responses to Environmental Insult

Eric Davies, Bratislav Stankovic

Abstract When plant tissue is abiotically injured by crushing, cutting, heat-wounding, electrical stimulation, or by several other means, the injured (perceiving) tissue generates electrical signals (action potentials and variation potentials) and transmits them to distant (responding) tissue. Here they evoke apparently disparate responses, such as callose formation, closing of plasmodesmata, stoppage of cytoplasmic streaming, inhibition of ribosome movement along messenger RNA (mRNA), and ultrarapid but transient accumulation of over 100 transcripts, which are degraded without being translated. These apparently disparate responses can be reconciled by one fundamental hypothesis that assumes that "the plant does not know what hit it" and thus "expecting the worst" mounts a holistic defense response against its most potent nemesis, a putative viral invasion. We postulate that the basis for this response is calcium influx into the cytoplasm via voltage-gated channels (action potential) associated with the microtubules, or via mechano-sensitive channels (variation potential) associated with microfilaments. The calcium interacts with calcium and/or calmodulin-dependent cytoskeleton-associated protein kinases. This causes the phosphorylation of myosin, which stops cytoplasmic streaming, and of elongation factor 2F, which slows elongation and termination and causes ribosomes to pile up on polyribosomes. This decreases protein synthesis, but protects preexisting "host" transcripts from degradation. The phosphorylation signal then passes into the nucleus, where it phosphorylates RNA polymerase II, which goes into overdrive (i.e., does not stop at accuracy checkpoints), thus causing the synthesis of large amounts of mismade mRNA. The mRNA is transported into the cytoplasm, where it is scanned (checked for accuracy) by ribosomes, and found to be incorrect. This surveillance mechanism stimulates ribonuclease activity, which degrades the free (non-polysome-associated), mismade RNA, but leaves the original, "host" transcripts unscathed since they are protected by ribosomes. The ribonuclease also (and here is the crux of the matter) attacks other free mRNAs, including viral mRNAs, so these are disposed of before they can be translated. Within minutes this reaction is over, cytoplasmic steaming resumes, translation continues, ribosomes are released and so can be used to translate new (correctly made) transcripts.

21.1 Introduction to the Hypothesis

There are several different kinds of electrical activities in plants, including action potentials (APs), variation potentials (VPs), voltage spikes or voltage transients, and rhythmic electrical activities but their role is far from understood (Davies 1987b, 2004; Davies et al. 1991). They all involve ion fluxes across membranes and bring about changes in membrane potential.

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It is assumed that they are a means of communication (i.e., intercellular signals) that can be generated and transmitted much more rapidly than can conventional chemical (hormonal) signals.

The two major types of putative signal are APs and VPs (Fig. 21.1). APs are defined as all-or-nothing, self-perpetuating signals, which are transmitted with essentially constant velocity and magnitude and are driven by voltage-gated channels (Zawadzki et al. 1991). Plant APs are very unlike neuronal APs, but are very similar to APs in other animal tissues (heart, epithelium). The neuronal AP can be thought of as the biological equivalent of a telephone, being designed for maximum rate of information transmission (velocity) and minimum amount of information leakage (security). This is done by having an ion-impermeable sheath over much of the neu-

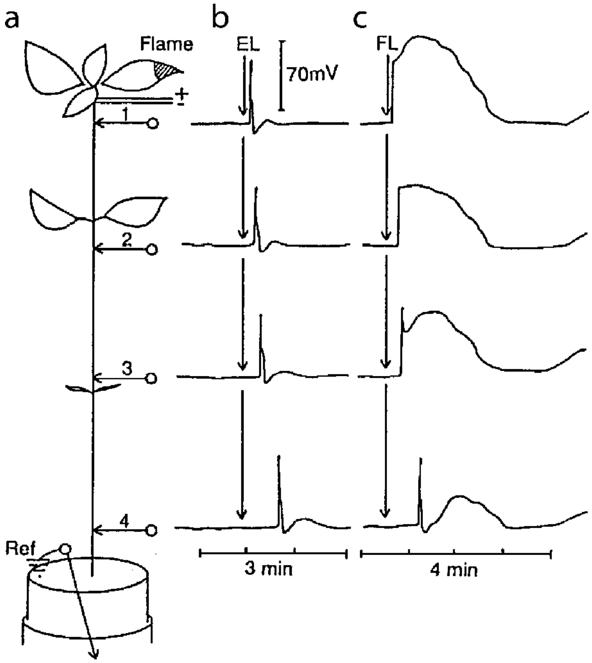

Fig. 21.1. Typical action potentials (*APs*) and variation potentials (*VPs*) measured in sunflower. **a** The plant was stimulated electrically (5 V for 1 s) at a point about 5 cm below the lowest petiole (±) or heat-wounded with a gentle flame applied to the tip of a leaf (*W*). Measuring electrodes (inserted silver wires) were placed along the stem, and a reference electrode was placed in the pot. The electrical responses to electrical stimulus are action potentials that are shown in **b**. The electrical responses to the heat-wounding stimulus are variation potentials that are shown in **c**. Note that electrical stimulation evoked a pure AP, while flame-wounding evoked a combined AP/VP, with the former "traveling" faster than the latter (Reproduced from Davies et al. 1991, with permission)

ron's length. This prevents the tissue through which it is passing being "informed", and also speeds up the rate of transmission by having the signal (ion flux, change in membrane potential) jump from "leak-point" to "leak-point." In contrast, APs in plants and in nonneuronal tissues can be thought of as megaphones, where signal velocity is reduced in order to maximize information spread, i.e., all the cells on the pathway (especially the phloem) are informed, and presumably modified, by the passing signal (Davies 1987a, 1987b; Zawadzki et al. 1991).

The VP differs considerably from the AP; it is not all-or-nothing, nor is it self-perpetuating (Stankovic et al. 1997c), rather it is a hydraulic surge, or possibly a transported chemical (Malone 1996) in the xylem evoking local changes in membrane potential in adjacent living cells. The rapid loss of tension in (dead) xylem elements is transduced into local changes in ion flux through mechano-sensory ion channels in the adjacent living cells (Stankovic et al. 1997b,c, 1998) or perhaps via ligand-activated channels if there is a chemical transported (Malone 1996). Thus, continuing with the information transmission analogy, the VP could be likened unto a radio signal being broadcast throughout the plant and "heard" on all the cells in the vicinity.

Regardless of the exact mechanism of transmission of APs and VPs, the responses they evoke must depend on either the ions traversing the membrane or the change in membrane potential, or both. Evidence suggests that both signals might involve calcium influx followed by chloride and potassium efflux. However, even if the same ions are involved, the downstream events might not be the same, since the flux of any ion will depend on the kind, location, number, connections, and other properties of the channel through which it passes. For instance, voltage-gated calcium channels involved in APs might open only transiently, be few in number, be located primarily in the phloem along the longitudinal axis and be connected to the microtubules (Thuleau et al. 1998), where they can interact with microtubule-associated Ca-binding proteins. In contrast, mechano-sensitive calcium channels involved in VPs might be open for longer periods, be abundant, be located primarily in living cells adjacent to the xylem around the entire cell and be connected to the microfilaments where they can interact with microfilament-associated Ca-binding proteins (Davies 1993; Wang et al. 2004). Finally, voltage-gated, mechano-sensitive, and especially ligand-activated calcium channels are likely to release some calcium into the soluble phase in the vicinity of the plasma membrane, where they can activate enzymes such as phospholipase C which will release another second messenger, IP3, from membranes, which, in turn, causes the release of more calcium from internal stores (Heilmann et al. 2001).

21.2 Evidence for Our Hypothesis

Since this paper is a hypothesis based on limited evidence, we will focus on those aspects of research conducted in our laboratory, supplemented where necessary by work from others.

21.2.1 Electrical Signals and Translation

We began to suspect a role for electrical signals and their involvement in wound-modulated gene expression in plants (aged etiolated pea epicotyls) when we found that polysome formation occurred very rapidly both above and below the site of wounding, implying that a rapidly generated bidirectionally transmitted signal, presumably an electrical signal, was involved (Davies and Schuster 1981). Since there was only a slight increase in the poly(A)RNA content of the polysome fraction, but a huge increase in the capacity to make protein both in vivo and in vitro, we assumed that wounding was increasing translation, presumably by increasing ribosome initiation (Davies and Schuster 1981).

This interpretation received support from subsequent experiments, which showed that the reinitiating ability in vitro of ribosomes isolated from wounded peas was markedly increased (Ramaiah and Davies 1985). However, it was subsequently disproved when the experiments were redone so that protein synthesis in vivo was conducted using totally intact (unwounded) tissue (Davies et al. 1986), rather than in tissue slices (wounded) as had been done earlier (Davies and Schuster 1981; Schuster and Davies 1983). Using the totally intact system we showed that, even though polysome formation was occurring, protein synthesis in vivo declined markedly (Fig. 21.2). This effect of wounding (formation of polysomes, inhibition of protein synthesis) could be mimicked in unwounded tissue by cycloheximide (Davies at al. 1986), a known inhibitor of elongation/termination. It became apparent that wounding was blocking ribosome movement along messenger RNA (mRNA), and we now suspect that this results from phosphorylation of the translation factor EF2, presumably via a calcium-dependent protein kinase.

21.2.2 Calcium, the Cytoskeleton, and Translation

We reasoned that if influxes of calcium occurred as part of the wound signal (AP), then cytoplasmic streaming should be inhibited, but to check

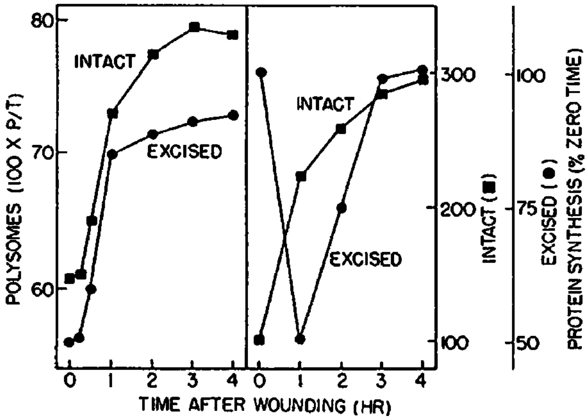

Fig. 21.2. Polysome formation and protein synthesis. Intact tissue – 2-cm-long aged epicotyls which had been excised 2 days earlier (to provide a cut surface for amino acid uptake and to permit recovery from the wound) were left undisturbed (unwounded), while others were nicked at the apex (wounded) for various time periods and then placed with their bases in labeled methionine for 30 min and then the basal 2 mm was extracted and assayed for protein synthesis. Similar samples were frozen in liquid nitrogen immediately after the end of the wound treatment and used for polysome isolation. Excised tissue – similar epicotyls were used, but the 2-mm basal piece was excised before the period of incubation and assay for polysomes. Note that similar polysome formation occurred in both tissues after wounding, but intact tissue exhibited a massive initial decrease in protein synthesis, while excised tissue exhibited an ongoing increase (Reproduced from Davies et al. 1986, with permission)

this we needed a system that would allow continuous monitoring of cytoplasmic streaming as well as ready uptake of exogenous compounds. For these studies we chose the aquatic plants, *Elodea* and *Vallisneria* (Davies 1990). We showed that wounding did, indeed, inhibit both protein synthesis (Fig. 21.3a) and cytoplasmic streaming (Fig. 21.3b) in these plants, as did treatment of unwounded plants with calcium plus ionophore, while removal of calcium relieved the inhibition.

Taken together these data suggest that wounding causes an increase in cytosolic calcium, which, in turn, inhibits both cytoplasmic streaming and protein synthesis. The question then became "Does cytoplasmic streaming depend on protein synthesis, does protein synthesis depend on cytoplasmic streaming, or are both processes co-dependent on some other event?" We found no evidence for either interdependence, but did find evidence for a possible co-dependence – both processes might be governed by protein phosphorylation.

It has been known for some time that phosphorylation of myosin inhibits cytoplasmic streaming in plants, while phosphorylation of EF2 inhibits

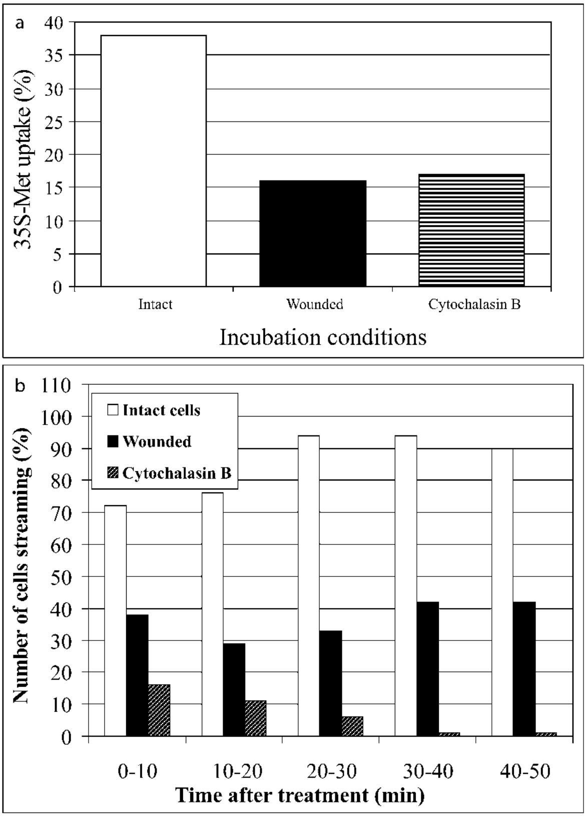

Fig. 21.3. Protein synthesis and cytoplasmic streaming in *Elodea*. **a** Leaves of the aquatic plant *Elodea* were either submerged intact in artificial pond water (*APW*), 2-cm pieces excised (wounded), or submerged intact into APW containing cytochalasin B and incubated for 1 h with labeled methionine to assay protein synthesis. Wounding and cytochalasin B caused similar inhibition (approximately 50%) of protein synthesis. **b** Similar samples were incubated in the absence of methionine and viewed continuously under the microscope at 10-min intervals to evaluate cytoplasmic streaming (Pfeifer and Davies, unpublished results). Note that wounding caused a consistent 45–50% inhibition of cytoplasmic streaming over the 50-min time period, whereas cytochalasin B caused an increasing (85–98%) inhibition over time, but removal of cytochalasin B relieved the inhibition (data not shown)

protein synthesis in animals by preventing ribosome movement along the mRNA (Ryazanov et al. 1988; Shestakova et al. 1991). But this still did not adequately explain the similar kinetics of inhibition and recovery of both processes. They were so tightly linked that they might be associated with the same structure – and if so, that structure was most likely the cytoskeleton. Already in the animal literature there was evidence for "detergent-resistant" polysomes that remained with the cell after all membranes had been removed, and so we set out to determine if such "cytoskeleton-bound

polysomes" exist in plants. They do. They are present in pea stems, pea roots, corn endosperm, and every other tissue we have examined (Davies et al. 1996), and they seem to be more translationally active than free polysomes (Davies et al. 1998). Indeed, the cytoskeleton seems to play a major role in the targeting, tethering, transport, and translation of mRNA (Davies et al. 2001).

21.2.3 Calcium Channels, the Cytoskeleton, and Transcription

Our work initially had been on translation (protein synthesis); research in this area seems to be less interesting (i.e., less funding is available) than research on transcription. This need for funding motivated us to change tracks and work on the model system for wound-evoked changes in transcription – the tomato plant. With this system we showed that both electrical stimulation and heat-wounding evoked the accumulation of proteinase inhibitor (PIN) transcripts, but only heat stimulus could evoke accumulation of calmodulin mRNA (Stankovic and Davies 1996, 1997a, 1997b).

While the bulk of workers looking at transcriptional responses to electrical signals wait several hours to make their first measurements, we conjectured that if plants generate ultrarapid signals, then they presumably exist in order to evoke ultrarapid responses. In our earlier experiments we isolated RNA within 15 min of wounding – and found some transcripts showed maximum accumulation at that time, before declining to basal levels and then rising again (Fig. 21.4). Interestingly, when we analyzed mRNA levels in polysomes (the currently translated mRNA), we found that most of the newly synthesized transcripts were not recruited into polysomes (i.e., never translated), but were degraded. These same transcripts usually increased again at a later time and these later-synthesized transcripts were recruited into polysomes for translation. Indeed, many transcripts showed this four-phase pattern: (1) a period of rapid accumulation, followed by (2) a period of equally rapid decline, followed by (3) a period of slow accumulation along with (4) their recruitment into polysomes (Fig. 21.4). When we shortened the wounding period to 2 min and used Northern blots (Davies et al. 1997), quantitative PCR (Coker et al. 2005) and DNA microarrays (unpublished data), we found over 20 transcripts reached a peak at that time point – some increasing as much as tenfold in 2 min. How could transcript accumulation occur so rapidly?

The simplest explanation for ultrarapid accumulation of transcripts is through enhancing transcription by activating the major enzyme responsible, RNA polymerase II (pol2). Evidence from the animal literature shows that pol2 normally adds 100 or more nucleotides to the nascent transcript,

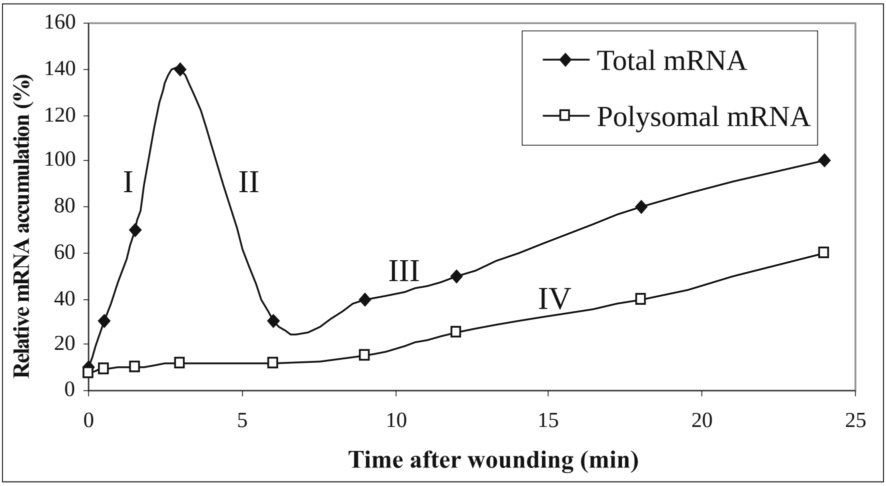

Fig. 21.4. Wound-induced systemic accumulation of total and polysomal messenger RNA (*mRNA*). Plants were wounded at zero time. Total and polysomal mRNA were isolated and the general pattern for 12 different mRNAs (including calmodulin, Rubisco SS, chloroplast mRNA-binding protein, bZIP DNA-binding protein) is shown. The four phases of transcript accumulation are indicated with *Roman numerals* (Davies et al., unpublished results). Note that the rapidly synthesized transcripts (phase 1) are degraded (phase 2) and not used in protein synthesis. The slowly accumulating transcripts (phase 3) are recruited into polysomes (phase 4) for protein synthesis

but then pauses to check for accuracy. However, when it is phosphorylated, it "goes into overdrive" (Landick 1999) and keeps adding nucleotides without checking for errors; thus, mRNA made by phosphorylated pol2 is virtually certain to be error-ridden. An alternative explanation for the ultrarapid increase in synthesis of mRNA is that pol2 copies one strand, then, rather than detaching and finding the appropriate start site on the sense strand, reveres direction and copies the nonsense strand (Cornellissen 1989). We have used primers for the antisense mRNA, but have not found any; thus, the most likely explanation for ultrarapid transcript accumulation is through speeding up (activation, phosphorylation) of pol2. This might take place via the same microfilament system used to phosphorylate myosin and EF2, since it has been shown that actin filaments penetrate directly into the nucleus and are associated with both pol2 and active transcription sites (Hofmann et al. 2004).

What would be the consequences of the cell making error-ridden mRNA? First, the ribosome would scan it for accuracy, find it in error and prevent other ribosomes from attaching to it. Activated ribonucleases (Abler and Green 1996; Gutierrez et al. 2002; LeBrasseur et al. 2002) would then degrade it in a wonderfully orchestrated surveillance mechanism (Kozak

Table 21.1. The first 10 min: early events following heat-wounding a leaf of a tomato plant

Duration (s)	Response	Effect	Reference
< 1	Loss of xylem tension throughout the plant	Systemic awareness of perturbation	Davies et al. (1991)
1–3	Mechano-sensing calcium channels open	Local awareness of perturbation	Davies (1993), Fisahn et al. (2004)
3–10	Calcium influx into the cytoplasm	Binds to calcium-binding proteins on MF	Roberts and Harmon (1992), Davies et al. (1996)
5–15	Activation of microfilament-linked CDPK	"Current" of phosphorylation along MF	Davies (1993), Wang et al. (2004)
5–15	Phosphorylation (inactivation) of myosin	Prevents cytoplasmic streaming	Davies et al. (1996)
5–15	Phosphorylation (inactivation) of EF2	Stops translation, ribosomes protect host mRNA	Ryazanov et al. (1988), Shestakova et al. (1991)
5–15	Phosphorylation (activation) of pol2	Makes error-ridden mRNA, goes into cytoplasm	Landick (2004), Hofmann et al. (2004)
30–120	Mismade RNA accumulates	Competes with viral RNA for ribosomes	Landick (2004)
120–500	Ca activation of RNase	Degrades unprotected viral and mismade RNA	Larkins and Davies (1973)
120–500	Disappearance of newly-made RNA	Newly made mRNA never translated	Fig. 21.4
3–10	Calcium influx into the cytoplasm	Binds to calcium-binding proteins near PM	Roberts and Harmon (1992)
5–15	Activation of phospholipase C	Releases IP3 from membranes	Heilmann et al. (2001)
20–60	IP3 passes from cell to cell	Calcium released from internal stores	Heilmann et al. (2001)
30–120	IP3/Ca stimulates RNA synthesis	Mismade transcripts accumulate	Salinas-Mondragón et al. (2001), York et al. (1999)
120–600	Activation of RNase	Degrades unprotected viral and mismade RNA	Larkins and Davies (1973)
120–600	Disappearance of newly made RNA	Newly made mRNA never translated	Fig. 21.4

MF microfilament, *CDPK* calcium-dependent protein kinase, *pol2* RNA polymerase II, *mRNA* messenger RNA, *PM* plasma membrane

1992; Beelman and Parker 1995; Hentzke and Kolozik 1999). Thus, in our heat-wounded tomato system, these rapidly synthesized transcripts are *not* recruited into polysomes; instead they are degraded and never used for protein synthesis. Thus heat-wounding of a mature leaf leads to a complex chain of events occurring systemically in the youngest leaf (summarized in Table 21.1).

21.3 Conclusions and Perspectives: The "Help! It's a Virus" Hypothesis

Why would a cell make a copious amount of mRNA, but degrade it without using it for protein synthesis. One possibility is that it has no function – but in this case one would expect it to be such an economic disadvantage that it would have been weeded out by evolutionary pressure. Another possibility is that the RNA, including viral RNA, is used for intercellular (interorgan) signaling. This does not seem to be the case, however, since virtually identical kinetics of transcript accumulation and disappearance take place in other parts of the plant, so there is nowhere else for preexisting mRNA to originate.

Our preferred hypothesis is that when the plant's integrity is compromised, the plant does not know what hit it, but mounts a defense against its most potent nemesis, a virus. Thus, essentially the instant its integrity is compromised, the wounded leaf informs the entire plant via the hydraulic surge and calcium channels open in all the cells capable of detecting such changes in turgor. Subsequent inhibition of cytoplasmic streaming will slow down virus movement within the cell; plugging of plasmodesmata will compartmentalize the virus and prevent its spread; slowing of ribosome movement along preexisting polysomes will cause ribosome "pile-up" on preexisting transcripts, thus protecting them from degradation by RNase; increasing the rate of transcription will cause an increase in error rate; flooding the cytoplasm with mismade RNA will lessen the availability of ribosomes to translate viral RNA as well as to stimulate breakdown of unprotected RNA. Within minutes, the plant has repelled its enemy, these (automatic?) defensive reactions cease, and now, having identified the specific nature of the recent insult, the plant generates an appropriate response and concludes "All is well with the world."

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22 Characteristics and Functions of Phloem-Transmitted Electrical Signals in Higher Plants

Jörg Fromm, Silke Lautner

Abstract Electrical signalling along the phloem has been studied in a number of species like maize, willow and *Mimosa*. It appears that sieve tubes with their large sieve pores are used to transmit information over long distances, while plasmodesmata serve as a means for the propagation of electrical signals over short distances between cells. By using the aphid technique the phloem pathway has been shown to transmit action potentials with a velocity up to 10 cm s^{-1}. With regard to the ion fluxes which create the conditions necessary for the generation of an action potential, we found that calcium influx as well as potassium and chloride efflux are involved. Some of their corresponding ion channels were identified. AKT2/3-like channels, expressed in the phloem and capable of mediating both uptake and release of K^+ in response to changes in membrane potential, were identified in several species such as *Arabidopsis*, maize and broad bean. Concerning physiological functions of electrical signalling, evidence was found for a link between the signals and photosynthetic response in *Mimosa*, apart from the regulation of rapid leaf movements. In addition, electrical signals in maize play a role in the regulation of phloem transport as well as in root-to-shoot communication of entire plants.

22.1 Introduction

Almost the entire chemistry of the neuromotoric system in animals is also available to plants. They do not possess nerves but neurotransmitters such as acetylcholine, cellular messengers like calmodulin, cellular motors, e.g. actin and myosin, voltage-gated ion channels and sensors for touch, light, gravity and temperature. This exciting cellular chemistry raises the question: Why do plants need cellular equipment similar to nerves? Although the degree of development and complexity of plant cells is not comparable to that of the nervous system in animals, plants seem to have developed a simple neural network within the phloem which serves the communication between plant organs over long distances. They have presumably developed such a system in order to cope in the best possible way with environmental stress factors. Having sensed environmental stimuli, the sensor region needs to transmit a signal to the responding region. The nature of this signal may be chemical (e.g. hormonal), hydraulic (e.g. pressure changes) or electrical (e.g. ionic). The electrical signals, in particular, have the capacity to rapidly transmit information over long distances. However, the conduction rate of most of the plant action potentials studied so far is

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in the range 0.01–0.2 m s^{-1}, i.e. much slower than the conduction velocity of action potentials in nerves, which is between 0.4 and 42 m s^{-1}. Similar to the velocities of action potentials in nerves are signals induced by pentachlorophenol in soybean, reaching a speed of 30 m s^{-1} (Volkov et al. 2000).

22.2 Signal Perception and Short-Distance Electrical Signalling

Environmental stimuli such as changes in light, temperature, touch or wounding can induce electrical signals at any site of the symplasm, and are transmitted via plasmodesmata to all the other symplasmic cells (van Bel and Ehlers 2005). For instance, mechanical stimulation of *Chara* cells generates the depolarization of the plasma membrane known as a receptor potential (Kishimoto 1968). Usually, the receptor potential lasts as long as the stimulus is present, being an electrical replica of the initial stimulus. If the stimulus is sufficiently great to cause the membrane potential to depolarize below a certain threshold, this will cause an action potential to be generated. It shows a large transient depolarization which is self-perpetuating and therefore allows the rapid transmission of information over long distances. To demonstrate intercellular coupling Spanswick and Costerton (1967) injected a current into a *Nitella* cell and managed to detect it using a microelectrode several cells away from the injected cell. Electrical coupling was also shown in a variety of tissues such as *Elodea* leaves and *Avena* coleoptiles (Spanswick 1972) as well as *Lupinus* stem phloem (van Bel and van Rijen 1994). These studies suggest that plasmodesmata are relays in a signalling network at the local level. If the information has to be transmitted to other parts of the plant further away, electrical communication via the phloem appears to be used (Fig. 22.1). Sieve tubes may be considered low-resistance pathways for the propagation of electrical signals over long distances. The few existing plasmodesmata between sieve elements/companion cells and phloem parenchyma cells (Kempers et al. 1998) may open up, making way for the electrical signals to move laterally from neighbouring cells into the sieve elements/companion cells. Therefore, the signalling cascade within the plant depends on the electrical conductance of plasmodesmata in a lateral direction and on the high degree of electrical coupling via the sieve pores in a longitudinal direction (van Bel and van Rijen 1994).

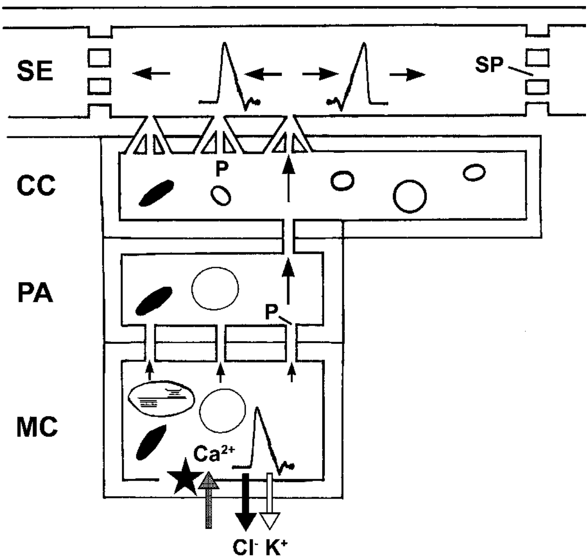

Fig. 22.1. Intercellular electrical communication in plants: Short-distance electrical signalling via plasmodesmata (*below*) and long-distance signalling along the sieve tube pathway (*above*). A stimulus like cold-shock or touch (*star*) induces calcium influx into a living cell, e.g. a mesophyll cell (*MC*). After the membrane potential is depolarized below a certain threshold level, an action potential is elicited by chloride and potassium efflux. The signal is propagated over short distances through plasmodesmal (*P*) networks and, after it has passed the few plasmodesmata between sieve element/companion cells (*SE/CCs*) and phloem parenchyma cells (*PA*), it will enter the SE/CC complex to be transmitted over long distances. Sieve pores (*SP*) with their large functional diameters present low-resistance corridors for the rapid propagation of electrical signals along the SE plasma membrane. Such signals can leave the phloem pathway at any site via plasmodesmata to induce certain physiological responses in the neighbouring tissue

22.3 Long-Distance Signalling via the Phloem

Apart from assimilate transport, long-distance signalling between various organs by physical and chemical signals travelling along the sieve tubes is a well-known process. Concerning electrical signals the transmission of action potentials along the plasma membranes of phloem cells is also an established phenomenon in plants which exhibit rapid leaf movements such as *Mimosa* (Samejima and Sibaoka 1983; Fromm and Eschrich 1988b). Previous studies using dye-filled microelectrodes reported that in *Mimosa* petioles the excitable phloem cells are small parenchyma or companion

cells (Samejima and Sibaoka 1983). Further studies showed that the sieve tubes also play a role in conducting action potentials. This assumption was confirmed in studies which used the aphid stylet technique, as first described by Wright and Fisher (1981) for *Salix exigua* and found to be suitable for studies on other species such as *Mimosa* (Fromm and Eschrich 1988b). Cooling the apical end of a *Mimosa* petiole generates a rapidly moving action potential which propagates basipetally through the sieve tubes. As for animal cells, an action potential is defined as a propagating, transient change in voltage with an all-or-none response, dependent on voltage-gated ion channels and capable of travelling through any living cells sharing common membranes. In contrast to action potentials, wounding, e.g. by flame-stimulation, causes the appearance of an irregular, so-called variation potential (Sibaoka 1966, 1969; Malone 1996). There is widespread agreement in the literature that the variation potential is not a self-propagating signal, but a local electrical response to the passage of chemical substances released from the wound site and propagated through the xylem by hydraulic dispersal. In contrast to action potentials, variation potentials are able to pass through a zone of killed plant tissue. In this context, the idea of a hydraulic conductance of excitation was re-examined in *Mimosa*, using a combination of electric and interferometric recordings (Tinz-Füchtmeier and Gradmann 1990). Since no significant correlation was detected between flame-stimulated electrical excitation and turgor changes, the results render a hydraulic conductance of excitation unlikely; but rather confirm the primary role of electrical events in rapid conductance of excitation in plants.

In the last 2 decades strong evidence has been accumulated that electrical transmission in sieve tubes also takes place in species that do not display readily visible reactions. In maize leaves both electrical stimulus and cold-shock trigger action potentials with amplitudes exceeding 50 mV which are transmitted without diminution in sieve tubes at velocities of 3–5 cm s^{-1} (Fromm and Bauer 1994). Sieve tubes also serve as a pathway for electrical signalling in root-to-shoot communication of water-stressed maize plants (Fromm and Fei 1998). Watering the root system after a drought period of 4 days induced a rapidly transmitted action potential. By contrast, water-stressing of roots through the addition of osmolyte to the root medium caused a different electrical signal to be measured in sieve tubes, indicating that the form of the generated signals depends on the type of stimulation. Not only in maize, but in the wounded tomato plant too, the pathway for systemic electrical signal transduction may be associated with the phloem (Rhodes et al. 1996).

22.4 Characteristics of Phloem-Transmitted Action Potentials

Almost all living plant cells have a resting potential with a net negative charge localized on the inner side of the plasma membrane, caused by unequal distribution of positive and negative ions via the membrane. Within the phloem, the membrane potential of companion cells is slightly more negative than that of sieve elements (van der Schoot and van Bel 1989). When a cell is stimulated, its electrical characteristics change significantly. The conformation of ion channels in the plasma membrane change in response to heat, touch, light or other environmental cues, leading to an increase or a decrease of the pore of the channels. This in turn controls the rate at which ions can migrate across the membrane and the size of the electrical potential difference. The latter changes from a negative to a more positive value (depolarization) during an action potential. Subsequent to depolarization the original resting potential is re-established (repolarization).

The ion transport processes which create the conditions necessary for the generation of an action potential were investigated intensively in members of the green algal family *Characaea* (Tazawa et al. 1987), indicating that calcium influx as well as chloride and potassium efflux are involved (Beilby and Coster 1979; Lunevsky et al. 1983; Spyropoulos et al. 1961; Oda 1976). The previously described ion displacements during an action potential were confirmed in trees by a method involving inhibitors of ionic channels as well as energy-dispersive X-ray microanalysis (Fromm and Spanswick 1993). Results from these studies lead to the conclusion that calcium influx as well as potassium and chloride efflux are involved in the propagation of action potentials (Fig. 22.1). In willow roots, an apparent efflux of anions and cations of 200–700 pmol cm^{-2} per action potential was calculated, using a vibrating probe in combination with the microelectrode technique (Fromm et al. 1997). Electrical stimulation of willow trees showed that the stimulus required to trigger an action potential depends on both its intensity and its duration (Fromm and Spanswick 1993). Increasing stimulus strength changes neither the amplitude nor the shape of the action potential, indicating that it conforms to the all-or-none principle. Stimulus sensitivity seems to depend on the individual plant because the excitability varied considerably although the trees were grown under identical conditions. Since the degree of excitability may also change in the course of a day and the season, it is likely that excitability depends on diurnal and seasonal rhythms (Zawadzki et al. 1991). As regards refractory periods, they were found to be much longer in plants than in animals, in the range between 2 min and 5 h (Zawadzki et al. 1991; Fromm and Spanswick 1993), while transmission velocities are between 2 and 10 cm s^{-1} (Fromm 1991; Fromm and Bauer 1994; Mancuso 1999), and in soybean up to 30 ms^{-1} (Volkov et al. 2000).

22.5 Ion Channels of the Phloem

The propagation of electrical signals along sieve tubes is achieved by opening and closing movements of ion channels in their plasma membranes. Since calcium, chloride and potassium fluxes are involved in the generation of action potentials in plants (Fig. 22.1), some of their corresponding channels were identified. Most of the work focuses on K^+ channels. The membrane potential of the sieve tubes, measured by means of the aphid technique, was shown to be dominated by K^+ conductance (Ache et al. 2001). Corresponding AKT2/3-like channels expressed in the phloem were identified in several species such as *Arabidopsis,* maize and broad bean (Marten et al. 1999; Deeken et al. 2000; Bauer et al. 2000; Lacombe et al. 2000). AKT2/3 is capable of mediating both uptake and release of K^+ in response to changes in membrane potential in a calcium- and pH-dependent fashion. Since AKT2/3 loss-of-function mutants (akt2/3–1) from *Arabidopsis thaliana*, lacking the phloem channels of the AKT2/3 type, possessed only half the sucrose content of the wild type, the authors assumed that the channel is involved in the loading of sugar into the phloem (Deeken et al. 2002). Furthermore, the akt2/3–1 mutant exhibited a reduced K^+ dependence of the phloem potential. Most likely, the channel is also involved in the generation of electrical signals, making it the subject of further studies. Concerning calcium, dihydropyridine-type Ca^{2+} channels were localized in the phloem of leaf veins from *Nicotiana tabacum* and *Pistia stratiotes* by immunolabeling techniques at the light and electron microscopic level (Volk and Franceschi 2000). The results indicate that sieve elements may be enriched with Ca^{2+} channels which may be involved in long-distance electrical signalling.

22.6 Functions of Electrical Signals in Higher Plants

There are numerous functions of short-distance electrical signalling via plasmodesmata in plants. For instance, insectivorous plants that live in nitrogen-depleted areas use electrical signals to capture insects in order to secure their nitrogen supply. When the outer *Drosera* leaf tentacles are touched by insects, the plasma membrane of the cells of the sensitive tip is depolarized (Williams and Pickard 1972a). Once depolarization exceeds a certain threshold, a series of action potentials is generated and propagated at a rate of 5 mm s^{-1} along the tentacle stalk. As soon as the base of the tentacle is reached, it is induced to wrap itself around the insect. Action potentials are also propagated to neighbouring tentacles and cause them

to wrap themselves around the insect too, so as to make a really secure trap (Williams and Pickard 1972a,b). Subsequently, secretory cells exude enzymes to digest the prey. Furthermore, the lobes of the trap leaf from *Dionaea* close when an insect strikes two hairs or one hair twice (Williams and Benett 1982; Hodick and Sievers 1989) to ensure that the trap only closes around live prey. The catching process starts with a release of calcium into the cytosol of the sensor cells (Hodick and Sievers 1988), induced by mechanical pressure. Subsequently, a depolarization signal is propagated at a speed of approximately 20 cm s^{-1}, causing the snapping of the trap (Sibaoka 1966). The fast closure of the trap is based on a snap-buckling instability, the onset of which is controlled actively by the plant (Forterre et al. 2005).

With regard to pollination, bioelectric potential changes were observed in the style of flowers, presumably transmitted via plasmodesmata down the style (Sinyukhin and Britikov 1967). Stimulation of the *Hibiscus* stigma by pollen, heat or cold-shock evokes electrical potential changes in the style which propagate towards the ovary at the rate of 1.3–1.5 cm s^{-1}, affecting its metabolism (Fromm et al. 1995). Self-pollination and cross-pollination induce signals which cause a transient increase in the ovarian respiration rate, indicating that the ovary metabolism was prepared for fertilization.

Long-distance electrical signalling via the phloem pathway is best known in plants performing rapid leaf movements. After touching a leaf of *Mimosa* an action potential is evoked and transmitted along the sieve tubes (Fromm and Eschrich 1988b; Fromm 1991) to cause shifts in ions and water between extensor and flexor cells in the pulvinus. As a result, the leaflets fold together and the petiole bends downwards making the leaf look dead and unappealing to a would-be herbivore. In the *Mimosa* petiole the vascular bundles are surrounded by a sclerenchyma sheath in order to restrict electrical signalling to the phloem. When a signal reached a pulvinus, it can be transmitted laterally via numerous plasmodesmata to the cells of the motor cortex. The latter possess voltage-gated ion channels which respond to electrical signals, causing ion influxes and effluxes associated with water fluxes that lead to leaf movements (Fromm and Eschrich 1988a–c). In addition, considerable amounts of photoassimilates are accumulated in the pulvini. Apart from the role of action potentials in the regulation of leaf movements, evidence was found for a link between electrical signalling and photosynthetic response in *Mimosa* (Koziolek et al. 2004). Following flame-wounding of a leaf pinna electrical signals travel rapidly into the neighbouring pinna of the leaf to eliminate the net-CO_2 uptake rate. At the same time the photosystem II quantum yield of electron transport is reduced. Two-dimensional imaging analysis of the chlorophyll fluorescence signal revealed that this yield reduction spreads acropetally through the pinna and via the veins through the leaflets (Koziolek et al. 2004). Further photosynthetic research

has to be done on the responsiveness of the various types of molecules that are involved in electron transport during electrical signalling.

Apart from *Mimosa*, consequences of phloem-transmitted electrical signals were also reported for tomato plants. They respond to wounding by the induction of proteinase inhibitor (PI) activity in parts of the shoot distant from the wound (Ryan 1990; Bowles 1990). The signal linking the wound site with the induction of PI activity was suggested to be either chemical, hydraulic or electrical. Wildon et al. (1992) provided evidence that the systemic wound signal is a propagated electrical signal. Their hypothesis is that the electrical signal passes from the wound site to the site of PI induction through electrically excitable cells that are coupled via plasmodesmata. Rhodes et al. (1996) studied the pathway of the electrical signal in tomato and were able to show that the phloem is involved. In addition, Stankovic and Davies (1997) found a definite relationship to exist between action potentials that were stimulated electrically and large, rapid increases in *pin* transcript.

Since long-distance transmission of electrical signals is associated with the phloem pathway it is obvious that action potentials have an effect on assimilate transport in the phloem. In maize leaves cold-shock as well as electric stimulation evoke action potentials which propagate via the sieve tubes away from the site of stimulation (Fromm and Bauer 1994). Simultaneously, phloem transport in distant leaf parts is sharply reduced, made visible by autoradiography at a distance of over 15 cm from the stimulation site. Evidence of a link between electrical signalling and the interruption of phloem translocation was found through a decrease in symplasmic K^+ and Cl^- concentrations during action potentials. According to Shiina and Tazawa (1986), who studied the effects of action potentials on the elongation growth in the stem of *Luffa cylindrica*, K^+ and Cl^- efflux from stimulated cells into the apoplast may reduce cell turgor and cause growth retardation. In maize, ion efflux during action potentials may also reduce the turgor of sieve tubes and trigger water efflux. Since water is the transport medium for assimilates, a reduction in water content will decrease phloem translocation (Fromm and Bauer 1994). However, the interruption of phloem translocation may also be attributed to reduced phloem loading because this process depends on the membrane potential and the K^+ concentration in sieve tubes. Therefore, repeated irrigations of the membrane potential during electrical signalling may have an effect on apoplastic phloem loading.

Further studies on the consequences of phloem-transmitted electrical signalling showed that action potentials are involved in the regulation of gas exchange in maize (Fromm and Fei 1998). In these plants, the CO_2 uptake and transpiration rate decreased strongly in drying soil. Subsequently, plants were watered and increases in CO_2 and H_2O exchange were

demonstrated to follow the arrival of an action potential in the leaves. These results therefore strongly support the view that phloem-transmitted electrical signalling plays an important role in the root-to-shoot communication of entire plants.

22.7 Conclusions and Future Perspectives

Although we cannot as yet fully image the genetic and metabolic complexity of plants we have managed to gain first insights into their multidimensional electrical communication system. Obviously, the signalling network responds to a variety of environmental factors which may be biotic or abiotic. Stimuli perceived in one part of the symplasm can be rapidly transmitted via electrical signals to other cells, tissues and plant organs. At the short-distance level, electrical coupling via plasmodesmata is a well-established phenomenon (van Bel and Ehlers 2005) and was demonstrated in a variety of tissues and species by conventional electrophysiology. After moving laterally through plasmodesmata, electrical signals are capable of entering the phloem network to effect fast communication over long distances (Fig. 22.1). The signals prefer to make their way through the low-resistance, longitudinally arranged sieve tubes.

Numerous examples exist with regard to the physiological or ecological functions of electrical signalling. *Mimosa* leaves look dead and unappealing to herbivores once the plant is touched. Insectivorous plants obtain their nitrogen by capturing and digesting insects. In ordinary plants that do not possess motor activity evidence was provided that action potentials may regulate a wide variety of physiological responses, including elongation growth (Shiina and Tazawa 1986), respiration (Dziubinska et al. 1989), water uptake (Davies et al. 1991), activation of PI genes (Wildon et al. 1992; Stankovic and Davies 1997), phloem transport (Fromm and Bauer 1994), gas exchange (Fromm and Fei 1998) and photosynthesis (Koziolek et al. 2004). For a better understanding of the complexity of electrical communication in plants, further studies involving novel methods with improved resolution will have to be conducted in the future.

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23 Long-Distance Signal Transmission in Trees

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Abstract Long-distance transmission of signals is a critical event in the life of trees. Many physiological studies have deduced that hormone-like substances together with hydraulic/electrical signals mediate this important function of the plant life. However the nature of the complex network of signalling in trees has remained essentially unexplored. Recent molecular and genetic studies offer new approaches to understanding the mechanisms underlying the transmission of signals.

23.1 Introduction

The ability to sense and respond to environmental parameters is one common feature of living being. This talent is particularly significant in land plants because of their necessity to manage all the environmental information and stimuli without escaping. Among plants, trees, for their necessity to respond in a short time to environmental stimuli despite the often big dimensions, are the most obvious and interesting subjects of study for the long-distance signal transmission.

Trees live in a continuously changing environment and although not all parts of the tree are exposed to the same stimuli at the same time, different parts of it respond in a coordinated fashion, demonstrating the existence of communication between various regions of the tree. For years, researchers have concentrated their efforts on the study of chemical (hormonal) signals in trees, neglecting the fact that plants also use and rely on electrical and hydraulic signals. In addition, it is especially in big plants such as trees that the need for signals other than hormones becomes more important. Considering the large leaf area of a tree, enormous amounts of chemicals would need to be synthesized and transported in order to respond to a signal coming from the roots. In contrast, both electrical and hydraulic signals consume no chemicals in their propagation.

Here, “long distance” will refer to signals running for distances that cannot be covered in reasonable times (a few hours) by diffusion in the aqueous phase, and that often have to cover several metres as in the case of root-to-shoot communication. For a long time, plant physiologists have presumed that signalling systems in plants must involve transport of “something” through the vascular system, effectively ignoring any other kind of signal

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like the electrical and hydraulic ones, whose importance is today increasingly confirmed by experimental results as is widely illustrated in many chapters of this book.

Trees may also transmit a multiplicity of signals to neighbouring organisms. These comprise the myriad of fatty acid derivatives, benzenoids, terpenoids and other scented substances emitted from flowers mainly to attract pollinators (Knudsen et al. 1993), but also the emission of isoprene (Loreto and Velikova 2001) or other volatiles from foliage or other vegetative parts of the trees (Paré and Tumlinson 1999). In the following pages, five different potential mechanisms for long-distance signalling in plants will be discussed:

1. Transmission of chemicals
2. Hydraulic signals
3. Electrical signals
4. Airborne flow of volatile messengers
5. Colour signals

23.2 Transmission of Chemicals

The roots of higher plants comprise a metabolically active and largely unexplored biological frontier. Their prime features include the ability to synthesize a remarkably diverse group of metabolites, and to adjust their metabolic activities in response to different abiotic and biotic stresses. Various experiments have shown that stomatal responses are often more closely linked to soil moisture content than to leaf water status, indicating a likely role of root-sourced chemical signals (e.g. abscisic acid, ABA) in the regulation of stomatal conductance in response to soil drying. Much is known about the role of ABA in closing stomata, as well as its production in dehydrating roots and its circulation in herbaceous species (Chaves et al. 2002). It is unclear, however, whether this is also true in mature trees, where long-distance transport of chemical signals from the roots to the shoots would be required (Jackson et al. 1995). In many instances the dynamics of ABA in trees is linked to changes in stomatal conductance (Blake and Ferrell 1977; Khalil and Grace 1993; Liang et al. 1997; Loewenstein and Pallardy 1998a,b; Maurel et al. 2004), but these results derive mostly from works with potted plants and/or in controlled conditions, and are species-specific (Wartinger et al. 1990; Tenhunen et al. 1994; Correia et al. 1995; Triboulot et al. 1996; Sturm et al. 1998; Niinemets et al. 1999). Particularly important

in much of the root-signal research in trees has been the use of split-root culture, where individual plants are grown with the root system divided among two or more separated soil volumes with independent moisture content. This situation shows that having a portion of the roots in dry soil can trigger strong stomatal closure even when shoot water potential does not decline, strongly supporting the hypothesis of a chemical signal derived from roots triggering stomatal closure (Gowing et al. 1990; Stoll et al. 2000; Augè and Moore 2002; Maurel et al. 2004).

23.2.1
From Where Does ABA Come?

It has been suggested that increased ABA delivery by the fraction of roots growing in the drying soil, rather than an increase in shoot xylem sap ABA concentration, is the signal for stomatal closure. Many studies have shown that, with increasing water stress, ABA is released from root tips into the transpiration stream and transported to the leaves, where it triggers stomatal closure (Khalil and Grace 1993; Triboulot et al. 1996; Fort et al. 1998). However, Fort et al. (1997) reported no increase in ABA delivery to the shoots of *Quercus* seedlings subjected to soil drying even though root xylem sap ABA concentration was increased and stomatal conductance was decreased. The importance of root-originated ABA in stomatal control, as well as the apparent sensitivity of stomatal conductance to root-originated ABA, may vary with genotype, within genotypes as a function of phenotypic plasticity, or with short-term changes in environmental parameters. Furthermore, apparent sensitivity of stomatal conductance to xylem sap ABA concentration decreases with time in water-stressed plants (Correia et al. 1995).

23.2.2
How Much ABA Is Involved in the Response of Trees to Drought?

According to field studies, a threshold response of stomata to xylem sap ABA concentration may exist, above which there are disproportionate increases in stomatal closure with xylem sap ABA concentration. This xylem sap ABA concentration was 500 nM in *Pinus* (Sturm et al. 1998) and *Ceanothus* (Tenhunen et al. 1994) but 200 nM in *Prunus* (Wartinger et al. 1990), indicating the existence of species differences in the stomatal control by ABA. Maximum ABA concentrations were only on the order of 100−150 nM in the study of Triboulot et al. (1996) and reached values of 300−350 nM in *Tilia* and 200–220 nM in *Populus* (Niinemets et al. 1999). Augé et al. (2000) found different values in xylem sap ABA concentration, from 600 to

2,000 nM during the season. The peak xylem sap ABA concentration found by Perks et al. (2002) during a period of severe drought (600 μmol m^{-3}) was of the same order of magnitude as previously reported in mature trees of other conifer species (Wartinger et al. 1990; Triboulot et al. 1996; Sturm et al. 1998), but no significant increase in ABA flux was recorded. Fluxes of ABA remained between 10 and 100 pmol m^{-2} s^{-1} in the work of Augé et al. (2000). Sap flow velocities recorded on a 41 year-old *Pinus* by Perks et al. (2002) are similar to those reported for mature conifer trees of other species (130 cm day^{-1} at 1 m and 240 cm day^{-1} at 9 m in water-stressed plants): data from Milburn (1979) for *Pinus* equate to a rate of 86 cm day^{-1} and data from Köstner et al. (1996) for *P. sylvestris* equate to a rate of 204 cm day^{-1}. During the period of severe drought, Perks et al. (2002) estimated that the time taken for a signal to travel from the roots to the crown increased to more than 6 weeks. These data support the conclusion of Schulze (1991) that "in conifers a root signal transported in the xylem may be too slow to be effective".

23.2.3 ABA and Xylem Sap pH

Some authors have suggested that *g* in herbaceous plants might be regulated by xylem sap pH (Thompson et al. 1997; Wilkinson and Davies 1997) or that stomatal sensitivity to ABA concentration might be modified by xylem sap pH (Jia and Zhang 1997; Zhang et al. 1997): pH gradients in the leaf control ABA distribution in the leaf and ABA concentration at guard cells, and thereby influence stomatal aperture. Results in trees cannot support this hypothesis, as stronger correlations of *g* with ABA concentration than with pH of xylem sap have been observed by Loewenstein and Pallardy (1998a,b). Augé et al. (2000) also found no significant association of *g* values with xylem sap pH.

23.3 Hydraulic Signals

Hydraulic pressure signals are propagating changes in water pressure inside plant tissues (Malone 1996). Plant tissues have plenty of hydraulic connections mainly composed of xylem, and they also provide a pathway for long-distance transmission of hydraulic signals (for an extensive review on hydraulic architecture of trees see Cruiziat et al. 2002). Pressure waves can be relatively quick and fast, as they can diffuse through the plant at the speed of sound (1,500 m s^{-1} in water), but, to be physiologically important,

a hydraulic signal must cause a significant change in turgor pressure inside a cell. As plant cells can be elastic, their turgor will change only when a strong influx (or efflux) of water occurs: the flux is strictly linked with the hydraulic capacitance of the cell. Thus, hydraulic signals must involve significant mass flow of water; for example, to increase the turgor pressure in leaf cells by 1 bar, a net water influx equivalent to 1–5% of the total volume of a leaf must occur (Malone 1996). Clearly, the kinetics of pressure change inside plant tissues; they are correlated to and depend on the magnitude and distribution of hydraulic resistances along the pathway. Leaf hydraulic resistances have been measured for a large number of tree species. Most of the resistance in the aboveground part of a tree is located within the leaf blade. For example, the leaf resistance expressed as a percentage of the total resistance between trunk and leaves is 80–90% for *Quercus* (Tyree et al. 1993a), around 80% for *Juglans* (Tyree et al. 1993b) and less than 50% for *Acer* (Yang and Tyree 1994). Measurements of leaf resistance in young apical and old basal branches of a *Fraxinus* tree have yielded contrasting results (Cochard et al. 1997). Most of the resistance was indeed located in the leaf blade in the apical shoots, but for older shoots, the resistance was mainly in the axis.

In addition, an important factor in hydraulic signal transmission is the hydraulic capacitance of the receiving tissue. There is considerable evidence that trees undergo seasonal and diurnal fluctuations in water content. These fluctuations can be viewed as water going into and out of storage. Water-storage capacity can be defined in different ways (Cruiziat et al. 2002). The relationship between water content and water potential is known as the hydraulic capacitance (Cw) of plant tissue and means the mass of water (ΔMw) that can be extracted per unit change in water potential ($\Delta\Psi$) of the tissue: Cw=ΔMw/$\Delta\Psi$. In general, hydraulic capacitance is difficult to measure, especially because it is not constant, but varies with the water potential.

The mass flow associated with hydraulic signals can be divided into two components (Malone 1996). The major one, characterized by a long axial pathway (xylem), has a volumetric flow rate approximated by the Hagen–Poiseuille law:

$$J_v \approx \frac{\pi r^4 \Delta P}{8\eta l}, \tag{23.1}$$

where J_v is the volumetric flow rate, r is the tube radius, ΔP is the pressure gradient, η is the kinematic viscosity of the fluid and l is the tube length. The viscosity of water inside xylem vessels varies from negligible values with dilute solutes to considerably higher values when concentrated solutes, like sugars, are present. It is important to note that J_v is proportional to

the fourth power of the capillary (xylem vessel) diameter. This means that a slight increase in vessel diameter causes a considerable increase in conductivity, and thus in hydraulic signal entity, when the other parameters have constant values. The smaller component is defined by a short radial pathway through cells at each end of the flow, which present more hydraulic resistance than the axial pathway.

In a transpiring plant, the water status of all tissues will firstly approach a dynamic equilibrium with their local xylem, and then with the entire plant. In fact, a change in the flux of water at any site can be transmitted throughout the xylem to any other site of the plant, thus affecting the turgor pressure of living cells. Examples of local changes are, for example, microvariations in the soil matric potential at root level or in light at leaf level (a cloud or wind movements).

The mechanisms by which stomata could sense changes in xylem pressure to adjust *g* remain largely hypothetical. It seems improbable that xylem pressure itself is the triggering parameter, so a variable correlated to xylem pressure during water stress must be identified. Cavitation is the abrupt change from liquid water under tension to water vapour (Cochard et al. 2002). As water is withdrawn from the cavitated conduit, vapour expands to fill the entire lumen. In a short moment of time (hours or less), air diffuses in and the pressure rises. The vessel then becomes "embolized" (air-blocked). The replacement of water vapour by air is the key point that makes embolism serious since air cannot be dissolved spontaneously in water as can water vapour. It is now clear that drought can induce cavitation and xylem embolism. Xylem cavitation is generally seen as a potentially catastrophic dysfunction of the axial water-conducting system (Salleo et al. 2000), but it may also act as a rapid hydraulic signal initiating the stomatal response, provided that it can be reversed without any major damage to water conduction. Because the onset of cavitation events in leaf blades was correlated with the onset of stomatal closure in *Laurus*, it has been suggested that stomata were responding to hydraulic signals generated by cavitation (Salleo et al. 2000; Nardini et al. 2001). However, in *Juglans* (Cochard et al. 2002), stomatal closure occurred before the onset of cavitation in leaf blades and midribs and only after 70% loss of conductance in the trunk.

23.4 Integration of Chemical and Hydraulic Signals

While it is difficult to reject the strong evidence for positive, root-sourced signals in trees, and the likely presence of ABA as the commonest signals for detecting water stress at soil-root level, it has also been hard not to

consider the leaf water potential (Ψ_l) to explain the full range of responses in stomatal conductance. Recent studies (Salleo et al. 2000) suggest that the stomatal response to drought is the result of the integration of hydraulic and chemical root-generated signals. For example, to link the dosage-response characteristics of the guard cells to ABA with observed xylem imports over a drought sequence, it is often necessary to assume increased sensitivity to ABA at low Ψ_l, an inherent merging of chemical versus hydraulic signalling concepts (Comstock 2002). Numerous reports detail a wide range of different stress treatments in various experiments which are associated with remarkably constant values of Ψ_l when comparing stressed and control plants. This constancy of Ψ_l is often cited as evidence against a hydraulic signal. Such a conclusion, however, implies that the diverse treatments are (1) as a side effect, causing large alterations in hydraulic conductance, (2) that ABA is having an entirely independent effect on stomatal conductance and (3) that these independent effects on hydraulic and stomatal conductance just happen to be so consistently well balanced and produce no measurable perturbation in Ψ_l. This is possible in some cases, but it seems an improbable explanation of such general behaviour. More likely hypotheses would be either that both chemical and hydraulic signals are operative and are integrated at the level of stomatal regulation, or even that hydraulic conductance itself is somehow being actively regulated. As has been pointed out previously (Tardieu and Davies 1993) it is often precisely in those species in which Ψ_l shows the least variation that a component of hydraulic signalling may be most clearly present. Nevertheless, statements that observed homeostatic conservation of Ψ_l during various treatments rules out hydraulic signals are still common in the literature. In describing the method by which leaf hydration controls *g* in woody plants, Saliendra et al. (1995) noted that both hydroactive and hydropassive processes are likely to be important and would involve both hormonal and hydraulic mechanisms. Correia et al. (1995), Thomas and Eamus (1999) and Augé et al. (2000) also noted the likely interaction of both hormonal and hydraulic influences in modulating *g*.

23.5 Electrical Signals

The wounding of a leaf or a part of the shoot is known to cause variations in the extracellular electrical potential measured with surface contact electrodes (van Sambeek and Pickard 1976; Shiina and Tazawa 1986; Wildon et al. 1989) or with platinum or silver wires inserted directly into the tissues (Roblin 1985; Zawadzki et al. 1995). Wound- or stimulus-induced electrical phenomena in plants consist of a so-called variation potential (VP) or

"slow-wave", which appears as a wave of negativity with a variable length, shape, amplitude and propagation velocity, capable of passing through dead tissues (Roblin and Bonnemain 1985; Malone 1996; Mancuso 1999) and linked with xylem tension (Mancuso 1999), and briefer and faster signals, called action potentials (AP), considered to be real self-propagated electrical signals (Pickard 1973; Malone and Stankovic 1991; Stankovic et al. 1997). It is also suggested that APs play a major role in intercellular and intracellular communication and for regulation of physiological processes at the molecular and the organism level (Davies 1987). Mancuso (1999) focused on differences between VPs and APs in their mechanisms of propagation. In fact, an AP cannot pass through a dead region of a tissue, is still present in plants at saturating humidity and the amplitude and propagation velocities of APs are fairly constant through the shoots.

Therefore, APs are propagating electrical signals and not merely a local response to a hydraulic dispersal. Though the pathway of APs is not completely clarified, intracellular recordings tend to locate the activity in the phloem parenchyma (Samejima and Sibaoka 1983; Fromm and Spanswick 1993) or in the phloem sieve tubes (Wildon et al. 1992; Fromm and Eschrich 1988a). Numerous papers have been published on the study of VPs and APs. For example, Davies (2004) reviewed the subject, answering to the question "What properties do electrical signals have that chemical signals do not have?" with four terms: *rapidity*, *ubiquity*, *information*, and *transience*.

Researchers have rarely focused on woody plants (*Tilia* and *Prunus*, Boari and Malone 1993; *Salix*, Fromm and Spanswick 1993; *Vitis*, Mancuso 1999) although it is in such plants that the need for rapid and efficient signals other than chemicals becomes more obvious. Instead, they have been mainly limited to studying the electrical signals in herbaceous or sensitive plants like *Mimosa pudica* and related species because of their visible response to the stimuli (Ricca 1916; Houwink 1935; Weintraub 1952; Sibaoka 1969; Fromm and Spanswick 1993; Malone 1994b; Koziolek et al. 2004).

Since electrical signals have been shown to be widespread in the plant kingdom (Pickard 1973), it is important to study the physiological processes that might be under their control. Recently, Koziolek et al. (2004) described *M. pudica* responses in light and dark reactions of photosynthesis that indicate electrical signals play an important role in triggering photosynthetic response across long distances within the plant, giving evidence for a link between electrical signalling and photosynthetic response of plants. Electrical signals also regulate assimilate partitioning in *M. pudica* (Fromm and Eschrich 1988b; Fromm 1991), showing that APs trigger sucrose unloading from the pulvinar phloem and cause the turgor-dependent leaf movements.

A strong interaction between APs and hormones has been shown in willow roots by Fromm and Eschrich (1993), with a physiological role in the gas exchange of leaves. So, APs may be evoked by plant hormones. The application of indole acetic acid (IAA) or isopentenyladenine (IPA) in the root medium triggered a propagating AP with an amplitude of more than 80 mV (IAA) or 50 mV (IPA): 3 min later the CO_2 uptake increased and the transpiration rate first slightly decreased and then increased (IAA) or definitely decreased (IPA). In contrast, ABA treatment resulted in a hyperpolarization on the membrane potential, explained by Fromm et al. (1997) assuming that K^+ leaves the cortex cells. Consequently, CO_2 uptake and transpiration decreased sharply after 3 min following stimulation. From these results, ABA-induced stomatal closure seems not to be based on the hormone itself, but on the ABA-induced electrical signal, which by membrane processes causes the stomata to close. Accordingly, Fromm and Eschrich (1993) proposed that information on the soil water content was electrically transmitted to the leaves. With the use of inhibitors of ion channels and energy-dispersive X-ray microanalysis it was demonstrated that influx of Ca^{2+} and efflux of Cl^- and K^+ are responsible for the current flowing during APs in willow roots (Fromm and Spanswick 1993). Efflux of negative ions would reduce the endogenous outward current at the basal elongation zone and enhance the endogenous inward current at the apical elongation zone.

Grapevine (*Vitis vinifera*) plants exhibit different forms of rapid communication after a stimulus. Following perception of environmental stimuli, hydraulic and electrical signals, travelling for long distances in the plant, are early events in the coordination of the whole plant or some of its organs. The velocity of propagation of the front of the main negative-going signal (VP) was 2.7 mm s^{-1}, while an AP propagated along the shoot with a velocity of about 100 mm s^{-1} (Mancuso 1999). Koziolek et al. (2004) showed that wound-induced electrical signals propagate with a velocity of 4–8 mm s^{-1} within different pinnae of a *M. pudica* leaf. Another type of signal that could be involved in the regulation of photosynthesis after wounding is a chemical signal spreading from the stimulation site through the phloem. The transport velocities in the phloem typically proved to be 50–100 cm h^{-1} (Canny 1975), which is much too slow to account for observed modifications in gas exchange. Also, the possibility of a chemical transport in the xylem can be ruled out because the stimulus was applied upstream within the leaf. Transport through the symplasm might be another pathway for a chemical signal, but the speed of this process (up to 15 µM m s^{-1} in higher plants; Tyree 1970) is even slower than the transport velocity in the phloem.

23.6 Airborne Flow of Volatile Messengers

Gaseous signal transmission in plants, from ethylene to nitric oxide, has a long and established history. The gaseous plant hormone ethylene was described in 1934, but since antiquity the fact that plants emit numerous volatile compounds from flowers, fruits and vegetative parts that exert activity on other organisms has been realized. For example, floral volatiles serve as attractants for species-specific pollinators, whereas the volatiles emitted from vegetative parts, especially those released after herbivore feeding, appear to protect plants by deterring herbivores and by attracting the enemies of herbivores (Kessler and Baldwin 2001; Pichersky and Gershenzon 2002). Leaves normally release small quantities of volatile chemicals, but when a plant is damaged by herbivorous insects, this quantity rapidly increases. An undamaged plant maintains small levels of volatiles as a constitutive chemical reserve, which includes monoterpens, sesquiterpens and aromatics (Markovic et al. 1996, in *Fraxinus*). In contrast, following insect damage, plants release a variety of newly formed volatiles from the damaged site: the composition of the volatile profile changes, because of a de novo synthesis. However, these compounds are not stored in the plant (Paré and Tumlinson 1999), but are quickly released in the surroundings. The metabolic cost of these phytochemical emissions can also be high. In particular, terpenoids are more expensive to manufacture per gram than most other primary and secondary metabolites owing to the need for extensive chemical reduction (Gershenzon 1994). It appears that volatiles need to be judiciously synthesized and safely stored, as increased synthesis can be costly and potentially toxic to the plant. However, decreases in terpene accumulation may make an individual plant more vulnerable to insect pest attacks or temperature stress. In addition to the release of volatiles at the site of herbivore feeding, analysis of volatile emissions from unharmed leaves of insect-damaged plants has established that there is a systemic response. Chemical labelling experiments showed the systemic volatiles are synthesized at the site of release, suggesting that a mobile chemical messenger is transported from the damage location to distal, undamaged leaves to trigger synthesis and volatile release, moving both acropetally (Jones et al. 1993) and basipetally (Davis et al. 1991). The observed signal transduction from sink to source leaves leads to the question of the nature of the systemic signal. Different signal types have been widely proposed, from electrical (Stankovic and Davies 1996), to chemical (Malone et al. 1994; Malone and Alarcon 1995; Rhodes et al. 1999) to hydraulic signals (Alarcon and Malone 1994). Recently, for the first time electrophysiological recordings were performed by Pophof et al. (2005) on single olfactory sensilla of *Cactoblastis cactorum*. Eight volatile organic

compounds emitted by *Opuntia stricta*, a host plant of *C. cactorum*, were identified using gas chromatography–mass spectrometry, β-caryophyllene being the major compound. Five compounds identified by gas chromatography in the headspace of *O. stricta* elicited responses in olfactory receptor cells of *C. cactorum*, nonanal being the most active compound and therefore a candidate attractant of *C. cactorum.*

23.7 Colour Signals

Colour change and colour pattern are powerful tools in plant–animal communication. The functional and evolutionary importance of colour signalling in animals has received great consideration in zoology, resulting in numerous theories and wide experimentation (Majerus 1998). In contrast, with the exclusion of studies on the colour importance for the attraction of pollinators to flowers (Chittka et al. 1999) and frugivors to fruit (Ridley 1930), the biological relevance of colour has been extensively underestimated in plant sciences. Yet, visual signals sent to animals are usually more efficient than olfactory signals on long-distance signalling, owing to the great influence of the environment on the diffusion of volatiles (Dobson 1994; Anderson and Dobson 2003).

One the most exciting colour signals produced by plants is the bright autumn coloration displayed by many deciduous trees. Why some tree species make this spectacular exhibition of colour is one of the most puzzling questions in tree biology. The usual explanation is that autumn colours are simply a secondary and mere side effect of leaf senescence. In autumn the degeneration of chloroplasts and the degradation of chlorophyll pigments in colourless low molecular products allows the red and yellow pigments (carotenoids and flavonoids) to appear from the background (Sanger 1971; Goodwin and Mercer 1983). This point of view, however, overlooks two important facts: many trees do not show any bright colouration in autumn and, more important, there are numerous pieces of evidence that colour change is also due to the synthesis of new pigments (Chang et al. 1989; Matile et al. 1992).

Two recent papers have challenged this interpretation by suggesting that these red and yellow leaf colours are an honest signal of tree's ability to defend itself against potential insect pests (Archetti 2000; Hamilton and Brown 2001). Hamilton and Brown's theory explains that the bright colour of autumn foliage is not just a side effect of chlorophyll reabsorption but acts as a signal, for aphids that are looking for places to lay their eggs, to indicate that the tree has invested heavily in chemical defence, and it is, therefore, not suitable for aphids. Hamilton and Brown (2001) predicted

that tree species which suffer greater insect damage invest more in autumn colour than less troubled species. Maples, for example, which exhibit one of the most impressive autumn displays, are some of the most heavily aphid infested species (Blackman and Eastop 1994).

Hagen et al. (2003) explored experimentally Hamilton and Brown's autumn signalling hypothesis in *Betula*. As predicted by the theory, early autumn colour change (i.e. more colourful trees in autumn) results in less insect damage the following spring. In addition, from an index of tree conditions (fluctuating asymmetry), they found a positive relationship between tree condition and colour signal intensity. Recently, Archetti and Leather (2005) published the first direct observation of a key assumption of the theory, that parasites avoid bright colours. By monitoring the colonization of the aphid *Rhopalosiphum padi* on individual tress of *Prunus padus* in autumn they were able to observe a strong preference of aphids for trees with green leaves and to demonstrate that aphids colonizing trees with green leaves develop better in spring than aphids colonizing trees with bright autumn colours, which is consistent with the main assumption of the theory.

23.8 Conclusions and Future Prospects

In the past year several exciting reports have suggested new models for the production and transmission of long-distance signals in physiological and developmental controls of trees. Nevertheless, we still have a long way to go to fully understand the complex roles of the different mechanisms. For example, the findings described in this review mark only the beginning of an interesting challenge to elucidate the complex regulatory network of chemical, hydraulic and electrical signalling and responses. Continuing advances in genomics, especially in the availability of mutants and genome sequences, along with developments in chip microarray technology, should cause rapid progress in this field. In addition, speaking about trees, we cannot forget to mention that grafting has been an essential technique in the discovery of long-distance signal pathways (e.g. root-to-shoot communication). We think that a refined grafting technique, also today, could be the key for allowing identification of alterations in gene expression which would give insights into long-distance signal transduction and gene functions.

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24 Electrophysiology and Phototropism

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Abstract Plants continually gather information about their environment. The conduction of bioelectrochemical excitation is a fundamental property of living organisms. Cells, tissues, and organs transmit electrochemical signals over short and long distances. The sensitive membranes in phloem cells facilitate the passage of electrical excitations in the form of action potentials. We have created a unique electrophysiological workstation that can effectively register this electrical activity in real time. It allows basic properties of electrical communication in green plants to be established. Our workstation has very high input impedance and a resolution of 0.01 ms. Excitation waves in higher plants are possible mechanisms for intercellular and intracellular communication in the presence of environmental changes. Ionic channels, as natural nanodevices, control the plasma membrane potential and the movement of ions across membranes regulating various biological functions. Some voltage-gated ion channels work as plasma membrane nanopotentiostats. Blockers of ionic channels, such as tetraethylammonium chloride and $ZnCl_2$, stop the propagation of action potentials in soybean induced by blue light and inhibit phototropism in soybean plants. Voltage-gated ionic channels control the plasma membrane potential and the movement of ions across membranes regulating various biological functions. These biological nanodevices play vital roles in signal transduction in higher plants. Tetraethylammonium chloride and $ZnCl_2$ block K^+ and Ca^{2+} ionic channels. These blockers inhibit the propagation of action potentials induced by blue light, and inhibit phototropism in soybean plants. The irradiation of soybean plants at 450 ± 50 nm induces action potentials with duration times of about 1 ms and amplitudes around 60 mV. The role of the electrified interface of the plasma membrane in signal transduction is discussed.

24.1 Introduction

Plants continually gather information about their environment (Ksenzhek and Volkov 1998). This conglomerate of information supports the maintenance of homeostasis. Environmental changes elicit various biological responses (Volkov 2000; Volkov and Mwesigwa 2001a,b). Plants synchronize their normal biological functions with their responses to the environment. The synchronization of internal functions, based on external events, is linked with the phenomenon of excitability in plant cells. The cells, tissues, and organs of plants possess the ability to become excited under the influence of environmental factors, referred to as irritants. The extreme sensitivity of the protoplasm to chemical stimuli is the basis for excitability; these signals can be monitored.

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Nerve cells in animals and phloem cells in plants share one fundamental property: they possess excitable membranes through which electrical excitations, in the form of action potentials, can propagate. Plants generate bioelectrochemical signals that resemble nerve impulses, and are present in plants at all evolutionary levels (Goldsworthy 1983). Prior to the morphological differentiation of nervous tissues, the inducement of nonexcitability after excitation and the summation of subthreshold irritations were developed in the vegetative and animal kingdoms in protoplasmatic structures.

The cells, tissues, and organs of plants transmit electrochemical impulses over short and long distances via the plasma membrane (Bose 1925; from and Bauer 1994; Volkov and Jovanov 2002). It is conceivable that action potentials are the mechanisms for intercellular and intracellular communication in response to environmental irritants (Bertholon 1783; Labady et al. 2002; Mwesigwa et al. 2000). Initially, plants respond to irritants at the site of stimulation; however, excitation waves can be distributed across the membranes throughout the entire plant. Bioelectrical impulses travel from the root to the stem and vice versa. Chemical treatment, the intensity of the irritation, mechanical wounding, previous excitations, temperature, and other irritants influence the speed of propagation (Shvetsova et al. 2001, 2002; Sinyukhin and Britikov 1967; Volkov et al. 2000, 2001, 2002, 2004, 2005).

The phloem is a sophisticated tissue in the vascular system of higher plants. Representing a continuum of plasma membranes, the phloem is a potential pathway for transmission of electrical signals. It consists of two types of conducting cells: the characteristic sieve-tube elements, and the companion cells. Sieve-tube elements are elongated cells that have end walls perforated by numerous minute pores through which dissolved materials can pass. Sieve-tube elements are connected in a vertical series known as sieve tubes. Sieve-tube elements are alive at maturity; however, before the element begins its conductive function, their nuclei dissipate. The smaller companion cells have nuclei at maturity and are living. They are adjacent to the sieve-tube elements. It is hypothesized that they control the process of conduction in the sieve tubes. Thus, when the phloem is stimulated at any point, the action potential is propagated over the entire length of the cell membrane and along the phloem with a constant voltage.

Electrical potentials have been measured at the tissue and whole plant level (Davies 1987). At the cellular level, electrical potentials exist across membranes, and thus between cellular and specific compartments. Electrolytic species such as K^+, Ca^{2+}, H^+, and Cl^- are actively involved in the establishment and modulation of electrical potentials. The highly selective ion channels serve as natural electrochemical nanodevices. Voltage-gated ion channels, as nanopotentiostats, regulate the flow of electrolytic species, and determine the membrane potential (Brown et al. 2005).

The huge amount of experimental material testifies that the main laws of excitability such as the inducement of nonexcitability after excitation and the summation of subthreshold irritations were developed in the vegetative and animal kingdoms in protoplasmatic structures earlier than the morphological differentiation of nervous tissues (Volkov 2000). These protoplasmatic excitable structures consolidated into the organs of a nervous system and adjusted the interaction of the organism with the environment.

Volkov and Haack (1995) studied the role of electrical signals induced by insects in long-distance communication in plants and confirmed the mechanism by which electrical signals can directly influence both biophysical and biochemical processes in remote tissues.

Bose (1925) has established the availability of a reflex arch in plants such as *Mimosa pudica*. When plants are excited sensory cells generate impulses which terminate at motor cells. The character of their distribution depends upon the physiological condition of plants. The signal from a beam of the sun is transmitted to the tissues of a stem at an extremely high speed and consequently the stem will curve toward the source of light. After excitation, the illuminated top of a stem causes an impulse to be distributed among the tissues (Volkov et al. 2005). When the impulse reaches motor cells, the stem bends. Thus, after electrochemical signals have reached the cell, deep cytophysiological reactions occur.

24.2 Phototropism and Photosensors

Light is an essential source of energy on which many of the biological functions of plants depend. The sun's radiant energy optimizes germination, photosynthesis, flowering, and other processes needed to maintain homeostasis. The first experiment on phototropism is probably lost in antiquity. Most likely someone noticed an indoor potted plant bending toward a window and rotated the pot 180° and then noticed later that the plant again bent back toward the window. The first scientists to really make progress in explaining phototropism were Charles and Francis Darwin (1888). They grew canary grass seedling and placed little caps of tinfoil or this glass painted black on the tips of the coleoptiles and determined what portion of the coleoptile had to receive light in order for phototropism to occur. Darwin (1888) found the tip was the light-sensitive part, but the bending also occurred well below the tip. Darwin (1888) hypothesized that an "influence" was translocated from the illuminated tip to the part where bending occurred.

Plants contain specific photoreceptors that perceive light ranging from UV to far-red light. Natural radiation concurrently excites multiple photoreceptors in higher plants. Specific receptors initiate distinct signaling pathways, leading to wavelength-specific light responses. Photoreceptors phototropins, cryptochromes, and phytochromes have been identified at the molecular level (Ahmad and Cashmore 1993; Ahmad et al. 1998; Casal 2000; Lasceve et al. 1999; Lin et al. 1996; Quail 1997; Reymond et al. 1992; Swartz et al. 2001).

Phototropin is a blue light (360–500 nm) flavoprotein photoreceptor responsible for phototropism, rapid inhibition of hypocotyls growth, stomata opening, chloroplast movements, and leaf expansion. The phototropins, such as phot1 and phot2, are a family of flavoproteins that function as the primary photoreceptors in plant phototropism and in intracellular chloroplast movements. Phot1, which is a plasma-membrane-associated member of this family, has two 12-kD flavin mononucleotide (FMN) binding domains LOV1 (light, oxygen, and voltage) and LOV2 within its N-terminal region and a C-terminal serine/threonine protein kinase domain. Phototropin, when activated by light, undergoes a conformational change. Phot1 and phot2 bind FMN and undergo light-dependent autophosphorylation. Phot2 is localized in the plasma membrane. It regulates phototropism and intracellular chloroplast movements. Phot1 and phot2 bind FMN and undergo light-dependent autophosphorylation.

Phytochrome is a bluish protein photoreceptor which regulates many aspects of plant development (Quail 1997; Smith 2000). Phytochrome A and phytochrome B perceive light as an environmental signal for the adaptation to fluctuating circumstances by essentially different manners in terms of effective wavelengths, required fluence, and photoreversibility. Phytochrome at its N-terminus contains a chromoprotein that can adopt two spectroscopically distinct, but interconvertible forms Pr and Pfr. Phytochrome is synthesized in the dark as Pr, the inactive form of the pigment. Pr absorbs red light (600–700 nm) and it is converted to Pfr, the active form of phytochrome. In turn, active Pfr absorbs far-red light (700–750 nm) and is converted to Pr. In vivo, Pr is synthesized at night and converted to Pfr, the active form, during the day. Then, the following night the reverse can occur and Pfr can be converted back to Pr. Hence, phytochrome enables plants to measure and adapt to temporal changes in light conditions. It also functions as a time-keeping mechanism. There are five forms of phytochromes which have been identified: phy A–phy E. Plant phytochromes are also light-modulated protein kinases that process dual ATP-dependent autophosphorylation and protein phosphotransferase activities.

Cryptochromes (cry1 and cry2) are flavoproteins of the family of photoreceptors responsible for photomorphogenesis (Cashmore et al. 1999). They perceive (UV-A) light as well as blue light (360–500 nm). Although

cryptochromes and phototropin share many similarities they have different transduction pathways. Cry1 plays a significant role in the synthesis of anthocyanin and the entrainment of circadian rhythms. Cry2 plays a part in the photoperiodic flowering and cotyledon expansion. Cryptochromes were found to be predominantly in the nucleus.

Blue light and UV irradiation influence the opening of the stomata (Folta et al. 2001). It is suggested that zeaxanthin is a blue/green light photoreceptor (Eisinger et al. 2000).

Phototropism is one of the best-known plant tropic responses. A positive phototropic response is characterized by a bending or turning toward the source of light. When plants bend or turn away from the source of light, the phototropic response is considered negative. A phototropic response is a sequence of the four following processes: reception of the directional light signal, signal transduction, transformation of the signal to a physiological response, and the production of directional growth response.

24.3 Electrochemical Circuits

The ends of a correctly constructed electrochemical circuit measuring the electrical potential difference must always have metals or conductors with identical chemical composition. This is usually achieved by simple connection of two metals by copper wires. The inclusion between two metal conductors of a third metal conductor according to Volta's law does not change the potential difference at the output of a circuit (Volkov et al. 1998). The potential difference in an electrochemical circuit at equilibrium is caused by the change of Gibbs free energy ΔG during the appropriate electrochemical reaction:

$$E = -\Delta G/nF \,, \tag{24.1}$$

where F =96,500 C is the Faraday constant and n is the number of electrons. The electrical potential difference of an electrochemical circuit at equilibrium (E) is the electromotive force. The value of nFE characterizes the maximum electrical work that can be received through an electrochemical circuit. Equation 24.1 is the basis for the calculation of the Gibbs free energy for different electrochemical reactions. The electrode potential at a given temperature and pressure is determined by the magnitude of the standard electrode potential and the activities of the substances taking part in the electrode reaction. The standard potential is a constant, which is specific for each electrode, while the activities of the reacting species may be different, depending on the concentration of the reaction medium.

The classification of electrodes is based upon the chemical nature of the substances participating in the electrochemical process. Electrodes of the first type are systems in which the reduced forms are metals of electrodes and oxidized forms are ions of the same metal. Electrodes of the second type are systems in which the metal is covered by a layer of low-solubility salts (or oxide), and the solution contains anions of these salts (for oxide – OH^- ions). The Nernst equation for electrodes of the second type can be written as

$$E = E^0 - \frac{RT}{nF} \ln a_{A^{n-}} , \tag{24.2}$$

where E^0 is the standard potential of an electrode of the second kind. As follows from Eq. 24.2 that electrodes of the second kind are anions reversible. The potential of an electrode of the second kind depends on the anionic activity of the sparingly soluble compound of the electrode material. The values of the potentials of electrodes of the second kind are readily reproducible and stable. These electrodes are often employed as standard half-cell or reference electrodes with respect to which the potentials of other electrodes are measured (Volkov et al. 1998). Of greatest interest in practice are reversible Ag/AgCl electrodes. Ag/AgCl electrodes consist of a piece of silver wire covered with a layer of silver chloride and immersed in an electrolyte solution containing chloride ions. Ag/AgCl electrodes are initially unstable; however, stabilization can be accomplished by placing two electrodes for 24 h in 0.05 M KCl solution and connecting a short circuit between them. Ag/AgCl electrodes should be stored in the dark and protected from light while in use. Ag/AgCl electrodes are sensitive to temperature and should be maintained at constant temperature (Ksenzhek and Volkov 1998).

24.4 Measuring of Action, Graded, and Variation Potentials in Plants

Two distinct classes of cell bioelectrochemical potential measurements exist. The intracellular action potential is measured with one electrode placed inside a cell while the reference electrode is situated in the conducting medium surrounding the cell. The extracellular action potential is measured with both electrodes in contact with the conducting tissue embodying larger groups of cells. In the latter case the signal observed is due to the depolarization–repolarization process in a group of cells. Measurements of these two types have been made in animal electrophysiology. The

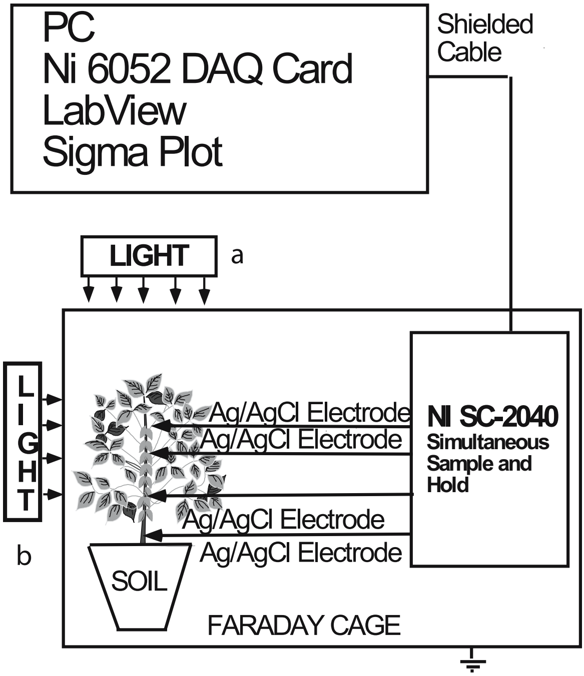

Fig. 24.1. Experimental setup for measuring electrical signals in green plants

well-known methods such as electromyograms (EMG), electrocardiograms (EKG) and electroencephalograms (EEG) are based upon measurements of the summed bioelectrical activity in large groups of cells.

Figure 24.1 shows the experimental setup for bioelectrochemical measurements inside a Faraday cage mounted on a vibration-stabilized table. An IBM-compatible microcomputer with a NI 6052E DAQ multi I/O plug-in data acquisition board (National Instruments) was interfaced through a NI SC-2040 simultaneous sample and hold (National Instruments). The multifunction NI 6052E data acquisition board provides a high resolution and a wide gain range and supports continuous, high-speed data acquisition. Single channels can be sampled at any gain up to 333,000 samples per second. Measuring signals were recorded using LabView (National Instruments) software.

A fundamental rule of sampled data systems is that the input signal must be sampled at a rate greater than twice the highest-frequency component in the signal. This is known as the Shannon sampling theorem, and the critical sampling rate is called the Nyquist rate. Stated as a formula, it says that $f_s/2 > f_a$, where f_s is the sampling frequency and f_a is the maximum frequency of the signal being sampled. Violation of the Nyquist criterion is

called undersampling and results in aliasing. The maximum sample rate is specified in samples per second, and not in hertz.

Ag/AgCl electrodes were prepared from Teflon-coated silver wire (A-M Systems) according to the method of Ksenzhek and Volkov (1998). The Ag/AgCl electrodes were identical; one electrode is referred to as the reference electrode, and the other as the working electrode. The reference electrode (–) was inserted in the stem or root of a soybean, whereas the working electrode (+) was inserted in the stem or a leaf of a soybean. The potential difference between the working electrode and the reference electrode is referred to as the variation potential in the plant. The mechanism of generation of a variation potential is not clear. Variation potentials measured in green plants, trees, and fruits typically have amplitudes from 20 to 140 mV.

24.5 Light-Induced Electrophysiological Signaling in Plants

The soybean was irradiated in direction A shown in Fig. 24.1 with white light in a Faraday cage for 2 days with a 12 h : 12 h light-to-dark photoperiod prior to conducting the experiments. Action potentials are not generated when the lights are turned off and on (Fig. 24.2).

After 1–2 min of irradiation, a change in the direction of irradiation from direction A to direction B generates action potentials in soybean

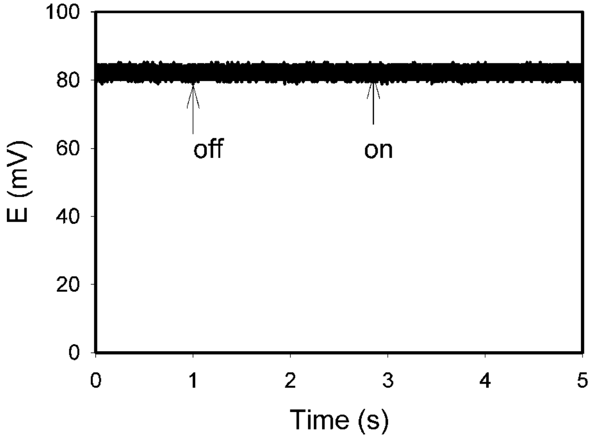

Fig. 24.2. Potential difference between two Ag/AgCl electrodes in the stem of soybean under irradiation (*on*) and in the dark (*off*). The distance between the electrodes was 5 cm. The soil was preliminarily treated with water every day. The volume of soil was 0.5 L. The frequency of scanning was 50,000 samples/s

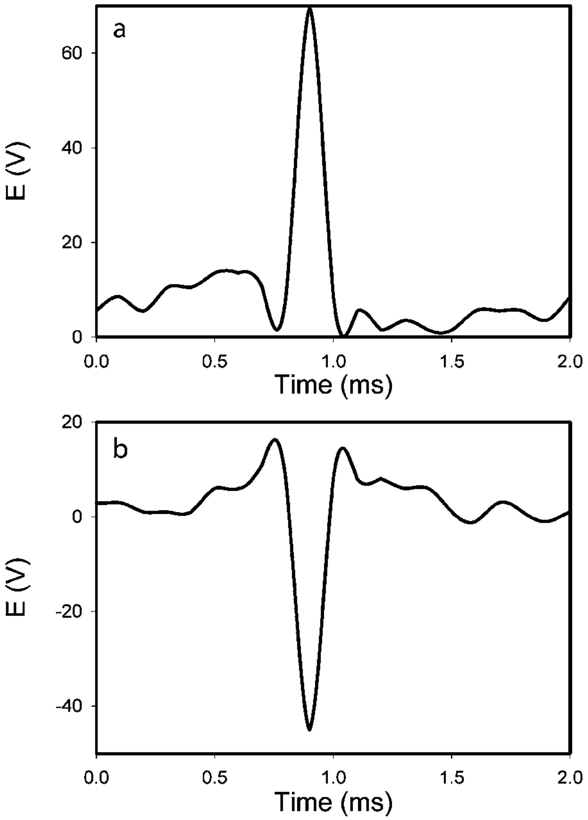

Fig. 24.3. Action potentials in soybean induced by irradiation at 450 nm in direction B as shown in Fig. 24.1. The irradiance was 8.5 $\mu E/m^2 s$. The distance between the electrodes was 5 cm. The soil was preliminarily treated with water every day. The volume of soil was 0.5 L. The frequency of scanning was 50,000 samples/s

(Figs. 24.3–24.5) depending on the wavelength of light irradiation. Irradiation at wavelengths 400–500 nm induces fast action potentials in soybean with a duration time of about 0.3 ms; conversely, the irradiation of soybean in direction B at wavelengths between 500 and 600 nm fails to generate action potentials. The action spectrum of light-induced action potentials is illustrated in Fig. 24.6. Irradiation between 500 and 700 nm does not induce phototropism. Irradiation of soybean by blue light induces positive phototropism (Fig. 24.7).

The sensitive membranes in phloem cells facilitate the passage of electrical excitations in the form of action potentials. The action potential has a stereotypical form and an essentially fixed amplitude – an "all-or-none" response to a stimulus. Each impulse is followed by the absolute refractory

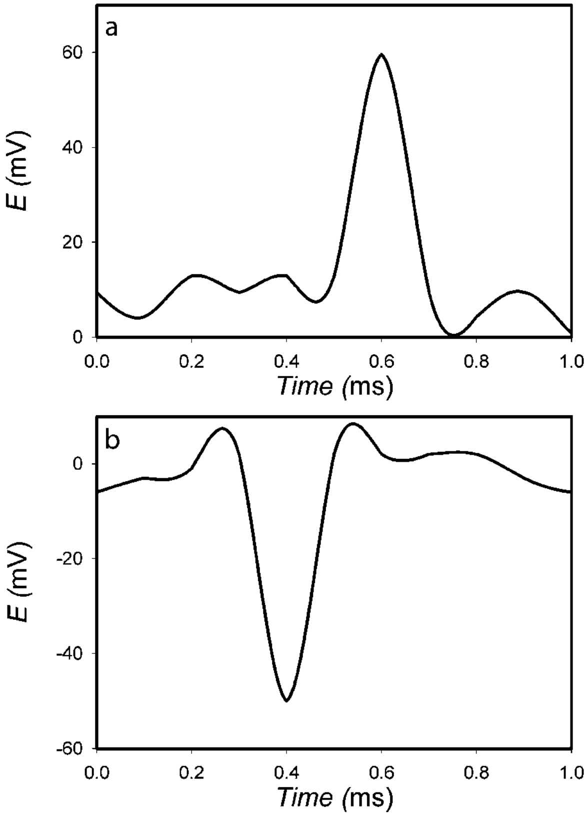

Fig. 24.4. Action potentials in soybean induced by irradiation at 470 nm in direction B as shown in Fig. 24.1. The irradiance was 8.5 μE/m^{2}s. The distance between electrodes was 5 cm. The soil was preliminarily treated with water every day. The volume of soil was 0.5 L. The frequency of scanning was 50,000 samples/s.

period. The fiber cannot transmit a second impulse during the refractory period. The integral organism of a plant can be maintained and developed in a continuously varying environment only if all cells, tissues, and organs function in concordance (Volkov et al. 2002).

These propagating excitations are theoretically modeled as traveling wave solutions of certain parameter-dependant nonlinear reaction-diffusion equations coupled with some nonlinear ordinary differential equations. These traveling wave solutions can be classified as single and multiple loop pulses, front and back waves, or periodic waves of different wave speed. This classification is matched by the classification of the electrochemical responses observed in plants. The experimental observations also show that under the influence of various pathogens, the shapes and speeds

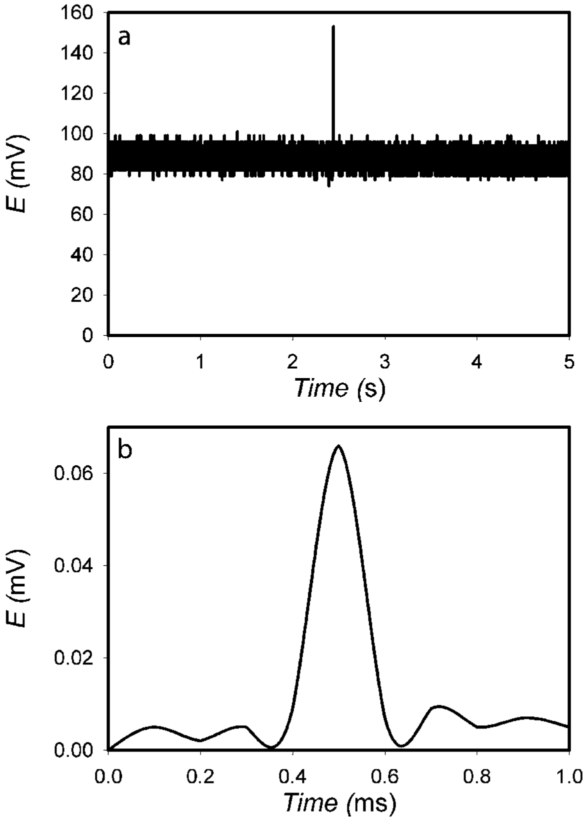

Fig. 24.5. Action potentials in a soybean plant induced by changing the direction of white light irradiation from direction A to direction B as shown in Fig. 24.1. The irradiance was 10 mE/m^{2}s. The distance between electrodes was 5 cm. The soil was preliminarily treated with water every day. The volume of soil was 0.5 L. The frequency of scanning was 50,000 samples/s

of the electrochemical responses undergo changes. From the theoretical perspective, the changes in the shapes and wave speeds of the traveling waves can be accounted for by appropriate changes in the parameters in the corresponding nonlinear differential equations (Volkov and Mwesigva 2001a).

Hodgkin and Huxley's membrane model accounts for K^+, Na^+, and ion-leakage channels in squid giant axons (Fig. 24.8a). The membrane resting potential for each ion species is treated like a battery and a variable resistor models the degree to which the channel is open.

Fromm and Spanswick (1993) discovered that the electrical stimulation of the plant is followed by ion shifts, which are most striking in

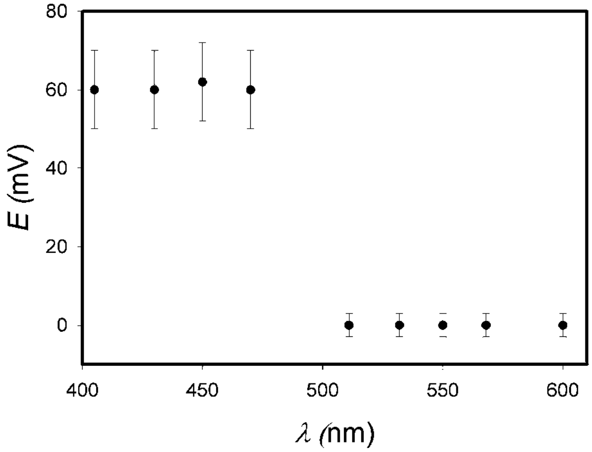

Fig. 24.6. Action spectrum: dependence of action potential amplitude on wavelength of irradiation

the phloem cells. The amount of cytoplasmic calcium increased slightly, while the content of K^+ and Cl^- was diminished after stimulation. Such evidence leads to the conclusion that Ca^{2+} influx as well as K^+ and Cl^- efflux are involved in the propagation of action potentials. In an axon there is K^+ and Na^+ transmembrane transport; conversely, in phloem cells the K^+, Ca^+, and more than likely H^+ channels are involved in this process (Fig. 24.8b).

Babourina et al. (2002) found that blue light induces significant changes in activity of H^+ and Ca^{2+} transporters within the first 10 min of exposure to blue light, peaking between 3 and 5 min. Blue light induced the opening of potassium and anion channels in plants and plant cells.

Some voltage-gated ion channels work as plasma membrane nanopotentiostats. Blockers of ionic channels such as tetraethylammonium chloride (TEACl) and $ZnCl_2$ stopped the propagation of action potentials in soybean induced by blue light and inhibited phototropism in soybean plants. Voltage-gated ionic channels control the plasma membrane potential and the movement of ions across membranes; thereby, regulating various biological functions. These biological nanodevices play vital roles in signal transduction in higher plants.

TEACl blocks voltage-gated potassium channels (Hille 2001). $ZnCl_2$ inhibits the function of calcium channels and possibly proton channels (Hille 2001). The propagation of action potentials with a constant amplitude and speed depends on the work of the ion channels. These blockers inhibit the propagation of action potentials induced by blue light (Volkov et al. 2005), and inhibit phototropism in soybean plants (Fig. 24.9).

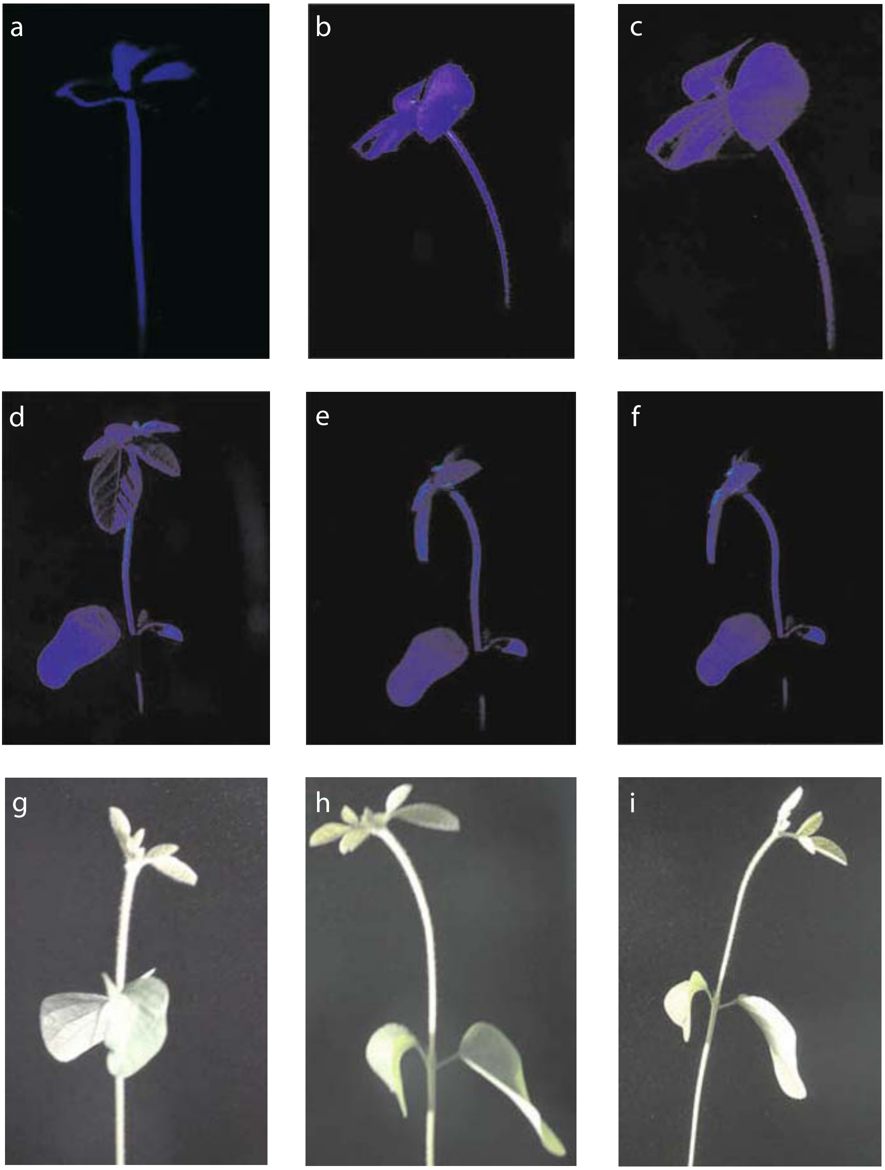

Fig. 24.7. Positive phototropism in a soybean plant at 450 nm (**a**–**c** light intensity 8.5 μE/m^{2}s), at 470 nm (**d**–**f** light intensity 9.8 μE/m^{2}s), and under 10 mW/cm^2 white light irradiation (**g**–**h**). Soybean was irradiated from the left side (**g**,**h**) or from the right side (**i**). Photographs were taken 10 s (**a**,**d**,**g**), 10 min (**b**,**e**,**h**) and 40 min (**c**,**f**,**i**) after beginning the irradiation

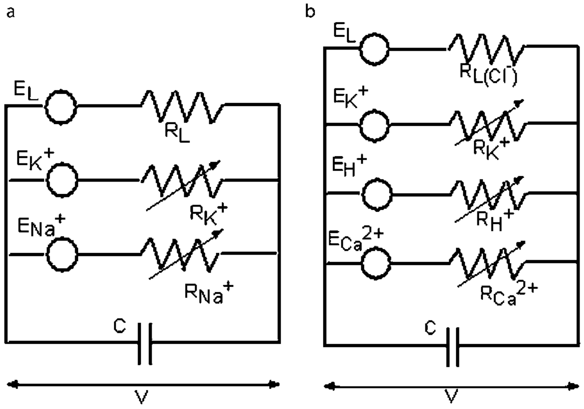

Fig. 24.8. The Hodgkin–Huxley (*HH*) equivalent circuit for an axon (**a**) and the modified HH circuit for sieve tubes in phloem (**b**)

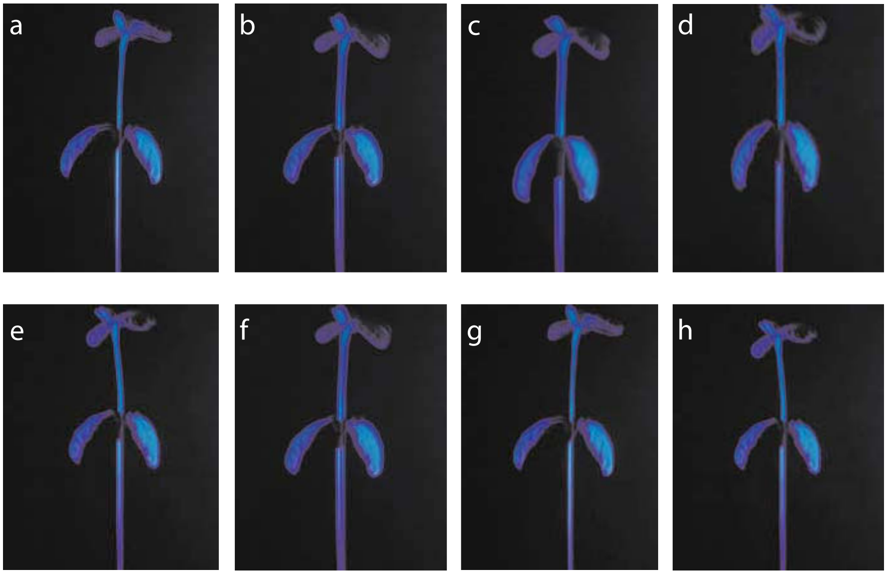

Fig. 24.9. Soybean plant after irradiation at 450 nm in direction B. The light intensity was 8.5 $\mu E/m^2 s$. Photographs were taken 10 s (**a**), 10 min (**b**), 20 min (**c**), 30 min (**d**), 40 min (**e**), 50 min (**f**), 60 min (**g**), and 70 min (**h**) after beginning of the irradiation. Soil around the soybean was treated with tetraethylammonium chloride 24 h before the photographs were taken

The duration of the action potential is not influenced by the location of the working electrode in the stem or leaves of the plant, or on the distance between the working and reference electrodes. Solitary waves, due to impulses generated by changes in environmental conditions, function as carriers of information in soybeans.

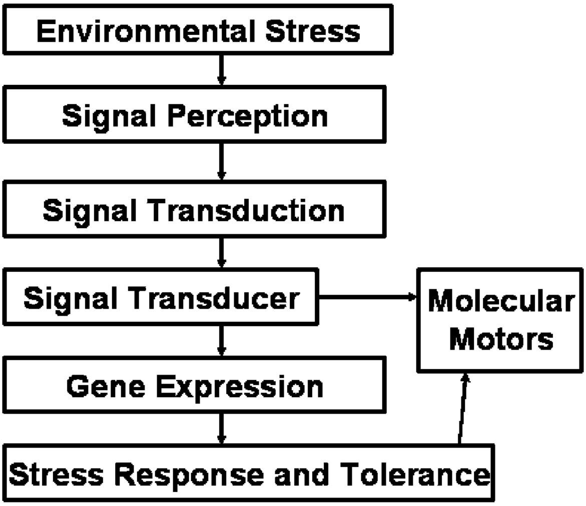

Fig. 24.10. Mechanism of biosignaling in green plants

All processes of life have been found to generate electrical fields in every organism that has been examined with suitable and sufficiently sensitive measuring techniques. The conduction of electrochemical excitation must be regarded as one of the most universal properties of living organisms. It arose in connection with the need for the transmission of a signal to an external influence from one part of a biological system to another. The study of the nature of regulatory relations of the plant organism with the environment is a basic bioelectrochemical problem, one that has a direct bearing on tasks of controlling the growth and development of plants. Figure 24.10 illustrates the mechanism of biosignaling in green plants.

Voltage-gated Ca^{2+} and K^{+} ionic channels of green plants can function as biological nanopotentiostats. The study of their electrical activity has tremendous medical and biological applications. Green plants are a unique canvas for studying signal transduction. It is the foundation to discovering and improving biosensors for monitoring the environment, detecting effects of pollutants, pesticides, and defoliants, predicting and monitoring climate changes, agriculture, and directing and quickly controlling the conditions influencing the harvest.

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25 Hydro-Electrochemical Integration of the Higher Plant – Basis for Electrogenic Flower Induction

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Abstract The integration of activity of *Chenopodium* plants on a hydraulic–electrochemical level is expressed by a diurnal rhythm in the resting membrane potential measured with contact electrodes. The membrane state could be gated by the energy state of cells. From earlier studies we compiled evidence in favour of a circadian rhythm in overall energy transduction producing a circadian rhythm in energy charge and redox state ($NADPH_2$/NADP). The ratio of metabolic coupling nucleotides would be relatively temperature independent and thus could fulfil the requirements for precise temperature-compensated time-keeping. The phytochrome photoreceptors, involved in photoperiodic control of development, could via changes in pyridine nucleotide pool sizes and changes in nucleotide ratios regulate transcription-translational loops by redox and phosphorylation controlled transcription factors. Spontaneous action potentials (APs) have been shown to correlate with turgor-controlled growth movements. The accumulation of spontaneous APs at specific times during daily light–dark spans were recorded, giving specific electrophysiograms, representative for flower-inducing and vegetative conditions. It is anticipated that hydraulic changes at the apex leading to flower initiation are mediated by a specific hydro-electrochemical communication between leaves, the shoot apex and the root system. These results have been used to substitute a flower-inducing photoperiod by specific timing of electric stimulation via surface electrodes.

25.1 State of the Art in Photoperiodic Control of Flowering in Short- and Long-Day Plants

Photoperiodic control of flowering involves perception of the critical photoperiod by the leaves, the production of the flower-inducing stimulus in the leaves, and its transduction to the shoot apex, where flower induction and evocation takes place. There is substantial evidence that the communication between leaves and the shoot apex involves frequency-coded signals in both organs and the change in their phase relationship upon flower induction.

To develop a coherent view on photoperiodic control in long- and short-day plants, the observations in Table 25.1 have to be considered, not least for the development of a hypothesis on electrogenic flower induction in short- and long-day plants.

To study interorgan communication between the signal-perceiving organ (leaf) and the target tissue (stem/apex) implied in the control of flowering,

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Table 25.1. Flowering of short-day plants (*SDP*) and long-day plants (*LDP*): essentials of the photoperiodic reaction

SDP and LDP show opposite reactions to a given photoperiod
Reactions result from coincidence or non-coincidence of light and dark phases of the photoperiod with corresponding phases of an endogeneous circadian rhythm
The main photoreceptors are the plant sensory pigment systems phytochrome and cryptochrome
Circadian rhythm and phytochrome have the same properties in SDP and LDP
Critical photoperiodical induction produces irreversible changes in the leaves of SDP and LDP leading to a common state both in SDP and in LDP (proven by grafting experiments)
There is no difference between SDP and LDP in their response towards a common inductor from a grafted leaf from an induced SDP or LDP

rhythmic kinetics of the stem extension rate (SER) and leaf movement (LM) were investigated in the short-day plant *Chenopodium rubrum* and in the long-day plant *Chenopodium murale*.

An undampened circadian rhythm in the SER was observed in continuous light. Total stem elongation depends on the precise cooperation of stem elongation of single internodes. In a specific experiment the first internode completed growth while the second and the third internodes both contributed to the total SER. The fraction of stem elongation due to the second internode declined, while the growth rate of the third internode increased. The two internodes thus displayed individual rhythms in the SER with the same phasing and a reciprocal change in amplitude, precisely controlled (Lecharny and Wagner 1984). As the stem grows, the internodes sustaining the undamped circadian rhythm in the SER move up.

To observe whole plant behaviour time-lapse photography was used showing rhythmic integration of the main shoot axis and side branches in rhythmic growth as well as in LMs. The SER was continuously monitored using an auxanometric system, while simultaneously analysing LM via a video system. Changes in organ surface potential were investigated using bipolar recordings with surface platinum electrodes (Fig. 25.1).

Cytoplasmic pH and Ca^{2+} concentration at the apical meristem were analysed using confocal laser-scanning microscopy and fluorescent dyes. Rhythmic LMs in *Chenopodium spp.* do not depend on differential turgor changes of flexor and tensor cells, as there are no pulvini at the basis of the petioles, but they are due to the timing of differential growth at the upper and lower surface of petioles and leaf basis. Detailed observation of time lapse movies clearly shows that the rhythmic folding up of leaves starts at the uppermost leaves surrounding the apical bud, progressing

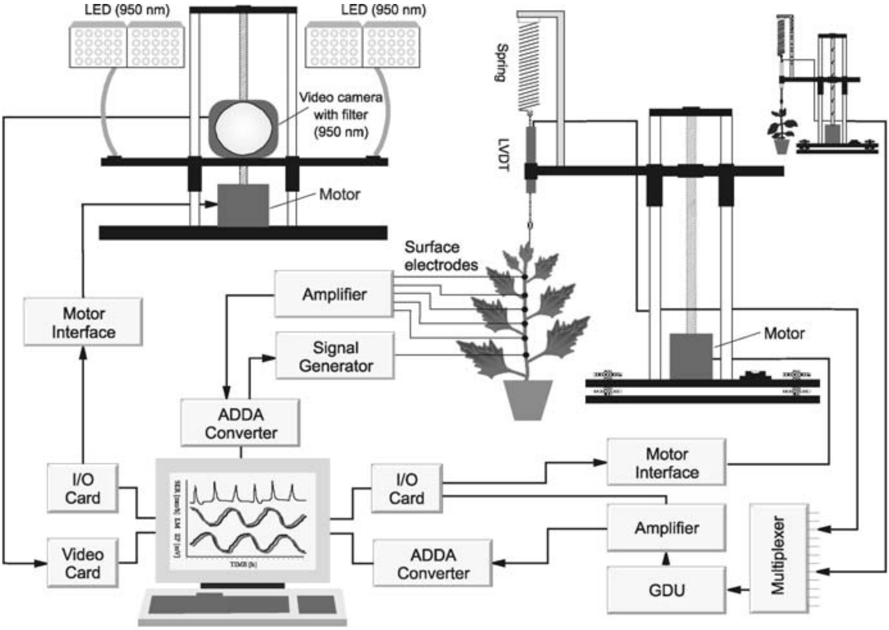

Fig. 25.1. Experimental setup. Measuring device for long-term recording of changes in electric surface membrane potential, leaf movements (*LM*) and stem elongation rate (*SER*). Video imaging at 950 nm for continuous monitoring of LMs in light–dark cycles. Linear voltage differential transformers (*LVDTs*) hooked to the plant stem with a spring-loaded constant pull of 1.5 g. Platinum electrodes are used together with a commercial contact gel for measuring and stimulation. Additional sensors monitor electromagnetic noise, temperature, light intensity and humidity

down the stem axis to the lowest still growing pair of leaves. The rhythmic LMs reflect rhythmic changes in hydraulics. Such changes in hydraulics are also obvious at the basal end of the plant from a circadian rhythm in root exudation (Fig. 25.2).

Control of cell volume and water relations at the plasma membrane most likely involves stretch-activated ion channels (Kloda and Martinac 2002; Lang and Waldegger 1997). Securing the integrity of the plasma membrane therefore seems imperative. Maintaining integrity of the plasma membrane might be the basis of hydro-electrochemical activity reflected in action potentials as discussed by Goldsworthy (1983).

Membrane potentials, being ubiquitous in all living cells, with the inside of the cell about 100 mV negative compared with the outside, are maintained by the activity of electrogenic ion pumps providing the energy for the active transport of many substances across the membrane. Depolarisation of cells leads to action potentials. Goldsworthy (1983) proposed that action potentials might have evolved as a mechanism for rapidly switching

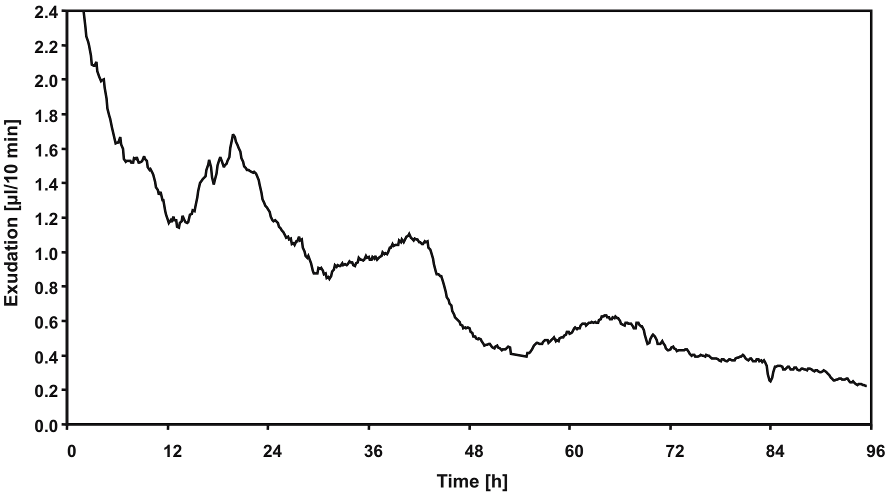

Fig. 25.2. Circadian rhythm of root exudation. Isolated root stock of a 5-week-old *Chenopodium rubrum* (ecotyp 184) under constant conditions (25 °C; 40% Hoagland's solution, dim white light) (Wagner et al. 1997)

off the membrane potential of cells to allow repair of a damaged cell membrane without losing too many ions from a localised injury. The generation of action potentials in plant cells would depend on the sensitivity of cells so that permeability of ions is increased by turgor-mediated mechanical deformation. Such changes depend on the metabolic activity of the cells leading to a change in turgor with subsequent changes in the activity of mechano-transductive ion channels (Kloda and Martinac 2002; Lang and Waldegger 1997).

Observed volume changes at the apex upon flower induction (Albrechtová et al. 2004) and the rhythmic changes in root exudation (Fig. 25.2) might be the basis for changes in the electrical activity at the root and shoot apical meristems. The observed diurnal rhythm in the resting membrane potential with significant changes for light-to-dark and dark-to-light transitions possibly reflects a change between a photosynthesis-driven (Fridlyand and Scheibe 1999; Karpinski et al. 1999) and a respiration-driven energy metabolism. Such a rhythmic change in energy metabolism as displayed in *C. rubrum* might be the essence of a circadian oscillator not only in higher plants but also in cyanobacteria (Ivleva et al. 2005).

This working hypothesis will be developed in more detail (Sect. 25.4). Redox and phosphorylation states are considered to be gating parameters of energy metabolism to adapt the development of living systems in daily light–dark cycles to avoid, for example, oxidative damage during light conditions.

These considerations support the view that action potentials and the circadian rhythmic organisation of energy metabolism are very early achievements in the adaptation of living systems to their natural environment.

25.2 Rhythms in SER as Markers of Photoperiodic Control and Interorgan Communication in a Long- and a Short-Day Plant

Both species (*C. murale* and *C. rubrum*) exhibit a circadian rhythm in the SER with period lengths of 24.3 ± 0.5 h (*C. rubrum*) and 27.5 ± 0.5 h (*C. murale*). Flowering plants show a significantly shorter period length in the SER of 23.44 ± 0.75 h (*C. rubrum*) and 26.6 ± 0.66 h (*C. murale*). The period lengths of LM mirror the kinetics in the SER, displaying similar increases of frequency in the flower-induced state. While in vegetative plants the kinetics of the SER and LM are 180° out of phase, this phase relationship is shifted after flower induction. Both parameters display clear movement and growth patterns with photoperiod-specific reactions to "light-on" and "light-off" signals. Flower induction correlates to a threshold value of stem growth of 0.6 (*C. rubrum*) and 4.0 (*C. murale*) for the ratio of the integral growth during the dark span over the integral growth in the light span. Two hours after the beginning of the critical dark period the pattern of cytoplasmic pH and Ca^{2+} concentration at the apical meristem has changed, possibly indicating the arrival of the inductive signal (Albrechtová et al. 2001; Walczysko et al. 2000).

25.3 Early Changes at the Shoot Apical Meristem During Flower Induction

The observations just discussed marked the beginning of a detailed kinetics analysis of early changes at the shoot apical meristem after the beginning of the inductive darkness. The cell physiological studies, paralleled by molecular studies, are well suited to closing the gap in recent investigations on the genetic control of flowering via a network of transcription factors, which led to the discovery of distinct signalling pathways predominantly in the model plant *Arabidopsis* (Blázquez and Weigel 2000; Reeves and Coupland 2000). In fact very little is known about the physiological basis of the morphological changes during flower induction and early flower development. However, recent studies demonstrated changes in carbohydrate

metabolism at the apex (Albrechtová and Wagner 2004) and in the shape of the apical meristem (Albrechtová et al. 2004), which led us to hypothesise on the possible involvement of a change in water status during an early phase of floral transition. Indeed, expression of a putative aquaporin *CrAQP* increases during flower induction at the apex and in leaves of *C. rubrum* (Albrechtová and Wagner 2004). Similar results were obtained in *Pharbitis nil* (A. Tretyn, personal communication). Furthermore, an application of an inhibitor of aquaporin activity to the apex delayed and partially inhibited flowering in *C. rubrum* (Albrechtová and Wagner 2004). On the basis of these observations, we proposed that changes in the water status at the apical meristem might play an important role in the initial phase of floral transition, leading to local changes in tissue tension caused by increased turgor, which in turn might activate mechano-transductive ion channels (Lang and Waldegger 1997). Local changes in tissue tension could directly influence organogenesis by affecting local properties of cell walls as proposed by Green (1994). Indeed, in *C. rubrum* the optical properties of cell walls at the surface of the apex change locally in the early phases of photoperiodic flower induction (Albrechtová et al. 2004).

On the basis of the observed early changes at the apex and of our measurements of changes in electrical activity during flower induction, we suggest that the signals for flowering might be transmitted from leaves to the apical meristem via hydraulic–electrochemical impulses. A similar mode of action was shown in systemic wound reactions (Wildon et al. 1992).

A diurnal (circadian) rhythm in the resting potential of the plasma membrane (Fig. 25.3) possibly reflects the daily change in photophile and skotophile phases (Bünning 1977). This rhythm has its origin in a circadian rhythm in energy metabolism and is probably the basis for circadian-rhythmic changes in sensitivity to signal perception, signal generation (e.g. action potentials) and signal transduction. It is proposed that the communication between plant organs (leaves, shoot apex, root) involves frequency-

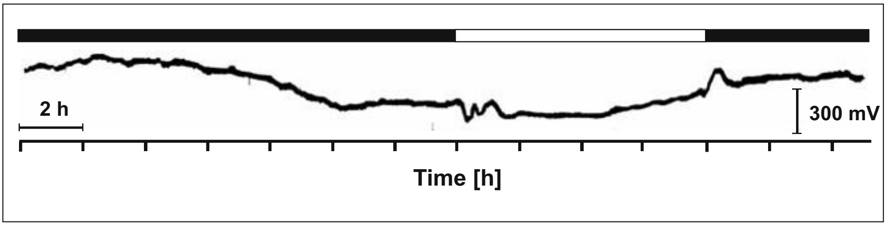

Fig. 25.3. Changes in resting surface membrane potential. Kinetics of the electric surface membrane potential in a light–dark cycle of 8 h : 16 h. Platinum surface electrodes were attached to a leaf petiole and the basipetal internode of a flowering plant of *C. murale* (see Fig. 25.1; Wagner et al. 1998)

coded (electric) signals (Wagner et al. 1998). Rhythmic integration over the whole plant possibly involves modulation of turgor pressure via stretch-activated ion channels and aquaporins, with concomitant changes in membrane potential (Fig. 25.3).

The perception of a flower-inducing dark period might lead to a change in electrochemical signalling between leaves and the stem and thus could represent "florigen". The involvement of action and variation potentials for integration of the whole plant was anticipated (Wagner et al. 1998). Signal arrival at the apex might trigger cytoplasmic changes in pH and Ca^{2+} concentration as secondary messengers in photoperiodic control of development (Love et al. 2004). Finally, the switch from the vegetative to the flowering state is a threshold response, systemic in nature and involving not only the apical meristem but also the axillary buds.

25.4 Evolution of Circadian Frequencies – Timing of Metabolic Controls

Considering metabolic control of timing in photoperiodism (Wagner and Cumming 1970; Wagner et al. 1975), it has to be kept in mind that evolution from prokaryotic to eukaryotic organisms was paralleled by a corresponding evolution in energy metabolism. From primary fermentation, energy conservation progressed to anaerobic photosynthesis and then to carbon dioxide fixation with acceptance of electrons by water and evolution of oxygen (Bekker et al. 2004). In a progressively oxygenic biosphere respiration developed with oxygen as the terminal electron acceptor. Evolving life was paralleled by the corresponding evolution of tropospheric O_2/CO_2 composition and feedback of oxygen on life processes via reactive oxygen and reactive nitrogen species, which as signalling molecules became crucial for control of development of prokaryotic and eukaryotic living systems. Adaptation to the seasonal variation in day length resulted in photoperiodic control of development with a circadian rhythm in energy conservation and transformation to optimise energy-harvesting by photosynthesis (Foyer and Noctor 2003; Wagner et al. 1975). Photosynthesis, on the other hand, acts as a metabolic regulator via redox signals (Oh and Kaplan 2000; Pfannschmidt 2003; Pfannschmidt et al. 2001; Sherameti et al. 2002; Zeilstra-Ryalls et al. 1998) in addition to specific photoreceptor systems like phytochromes and cryptochromes. Finally, redox control integrates rhythmic gene expression in prokaryotes (Ditty et al. 2003; Dvornyk et al. 2003; Rutter et al. 2001; Tomita et al. 2004), as well as in chloroplasts, mitochondria and the nucleus of eukaryotes (Forsberg et al. 2001; Tron et al. 2002).

The circadian rhythmic cell (cyanobacterial and eukaryotic) is considered to be a hydro-electrochemical oscillator (Wagner et al. 1997) synchronised by the daily light–dark cycles, with temporal compartmentation of metabolism and a network of metabolic sequences to compensate for oxidative stress in adapting to the light environment, e.g. by separating nitrogen fixation from photosynthetic oxygen production (Sherameti et al. 2002).

25.5 Circadian Rhythmic Organisation of Energy Metabolism in *C. rubrum* and the Gating of Photoreceptor (Phytochrome) Action

In *C. rubrum* a circadian rhythm in overall energy transduction has been observed. The rhythm results from an oscillatory network between glycolysis and oxidative phosphorylation, coupled to photophosphorylation. This network produces a circadian rhythm in adenylate energy charge and redox state ($NADP/NADPH_2$). The nucleotide ratios themselves could act as rate effectors in compartmental feedback and thus fulfil the requirements of precise temperature-compensated time-keeping (Wagner et al. 1975,

Table 25.2. Rhythmic organisation of metabolism in *Chenopodium rubrum* (the period lengths of the subpeaks are in *parentheses*) (Complemented after Bünning 1977)

Phenomenon	Period length (h)
Photoperiodic light sensitivity	30
Betacyanine accumulation	24–30 (15)
Betacyanine turnover	24–30
Adenylate kinase activity	30 (15)
Energy charge (ATP + 1/2 ADP/ATP + ADP + AMP)	21–24 (11–13)
$NADPH_2$/NADP ratio	21–24
Dark respiration	21–24
Chlorophyll accumulation	15
Net photosynthesis	15
Triose phosphate dehydrogenase activity ($NADH_2$; $NADPH_2$)	15
Malate dehydrogenase activity	12–15
Glutamate dehydrogenase activity	12–15
Glucose-6-phosphate dehydrogenase activity	12–15
Gluconate-6-phosphate dehydrogenase activity	12–15
Pyridine nucleotide, pool size [$NAD(H_2)$; $NADP(H_2)$]	12–15 (6)
Turgor-controlled growth phenomena	
Stem extension rate	23–27
Leaf movement	23–27

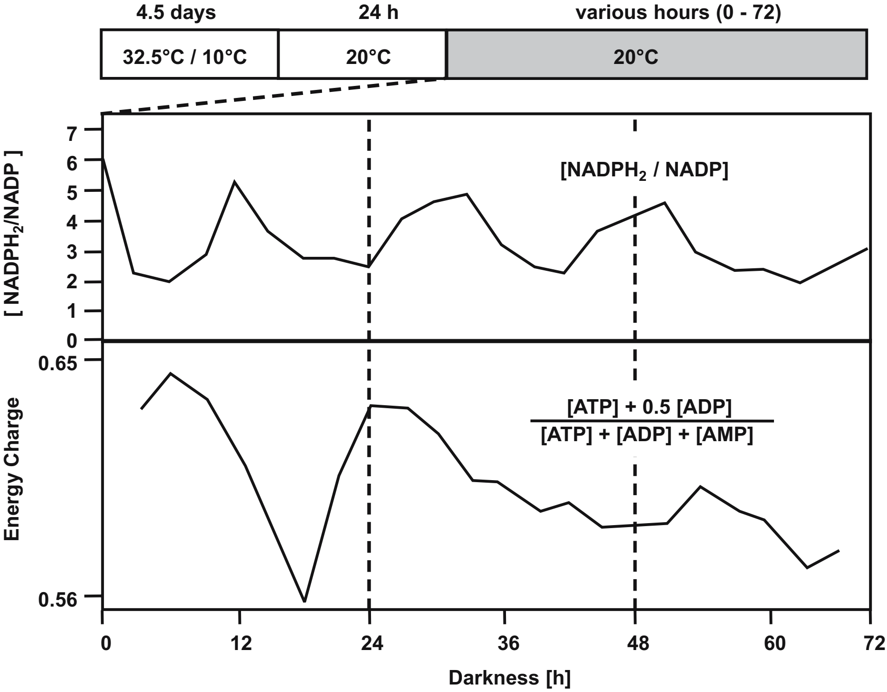

Fig. 25.4. Circadian rhythm of $NADPH_2$-to-NADP ratio and of "energy charge" of *C. rubrum* seedlings. The seedlings were germinated in constant light and alternating conditions of temperature for 4.5 days, thereafter transferred to constant 20 °C for 24 h followed by a dark period of varied duration (3-h increments) (Wagner et al. 1975)

1983, 1998). In *C. rubrum* there are semicircadian oscillations with specific phasing in enzyme activities involved in glycolysis, photosynthesis and respiration resulting in a circadian rhythm of adenylate energy charge and redox state (Table 25.2, Fig. 25.4).

There is an inverse phase relationship between glyceraldehyde-3-phosphate dehydrogenase (GPD) linked to photosynthesis (NADP-GPD) and the enzyme linked to glycolysis (NAD-GDP) (Fig. 25.5). Adenylate kinase, a key enzyme in energy dissipation displays circadian/semicircadian oscillations in activity. The enzyme exists as isoenzymes in chloroplasts, mitochondria, the cytoplasm and the nucleus. The enzyme activities are modulated by light (phytochrome, cryptochrome), glucose feeding and photoperiod and thermoperiod (Figs. 25.6–25.8).

The rhythm in enzyme activities may be amplitude-modulated by phytochrome. The phytochrome action is gated by the phasing of the endogenous rhythm reflecting a sequence of photophile and skotophile phases as conceived by Bünning (1977) for the functioning of the physiological clock (Figs. 25.9 and 25.10).

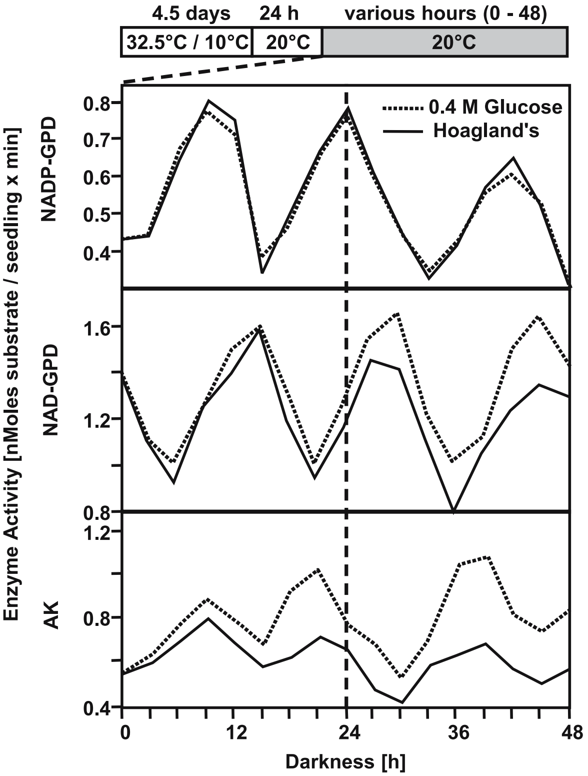

Fig. 25.5. Time course of adenylate kinase (*AK*), NAD- and NADP-glyceraldehyde-3-phosphate dehydrogenase (*GPD*) activity during darkness. *C. rubrum* (ecotype 184/68) seedlings germinated in alternating light intensity and temperature for 4.5 days. Thereafter constant 20 °C, 6,000 lx fluorescent white light for 24 h followed by a dark period of varied duration (3-h increments). The enzyme activities were measured at the end of each respective dark period. The dark period medium was either Hoagland's solution or 0.4 M glucose in Hoagland's solution under sterile conditions. There are semicircadian oscillations with an inverse phase relationship between photosynthetic NADP-GPD and glycolytic NAD-GPD. Glucose feeding in darkness has no effect on NADP-GPD but stimulates the other enzyme activities (Frosch et al. 1973)

25.6 Hydraulic–Electrochemical Integration of the Whole Plant

The integration of metabolic activity of *C. rubrum* plants on a hydraulic–electrochemical level is represented by a diurnal rhythm in compound surface-membrane potential (Fig. 25.2). Spontaneous surface-membrane action potentials could be shown to correlate with turgor-controlled hydraulic growth movements of leaves and stem extension (Fig. 25.11), which are controlled by the photoperiod. Recorded electrophysiograms can be

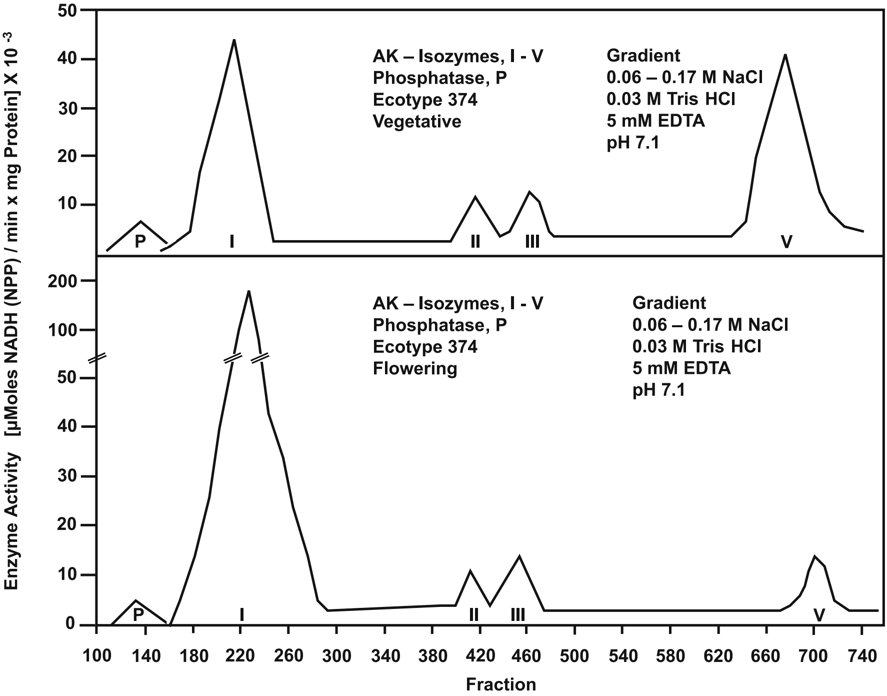

Fig. 25.6. Pattern of AK isozymes from *C. rubrum* (ecotype 374) after chromatography on (diethylamino)ethyl (*DEAE*) cellulose. *Top* Seedlings grown on Hoagland's solution with 0.5% agar at 6,500 lx continuous white fluorescent light for 24 days. *Bottom* The same conditions with one inductive dark period of 12 h, 5.5 days after sowing. The abundance of the different isozymes in the cellular compartments changes after flower induction; in particular the relation between chloroplasts (AK V) and mitochondria (AK I). Isozyme AK IV (nucleus) was not detected (Wagner et al. 1983). *AK I* mitochondria, *AK II* cytoplasm, *AK III* chloroplasts, *AK IV* nucleus, *AK V* chloroplasts, *P* phosphatase

used for the control of development, i.e. via electrogenic induction of flowering by DC pulses (Wagner et al. 2004).

At the apex, photoperiodic conditions inducing flowering have been studied at the cellular level in *C. rubrum* (Sect. 25.3). There are very early changes in calcium and pH patterning (Albrechtová et al. 2003) and carbohydrate metabolism, which could lead to an increase in osmotic pressure in the cells of the apical meristem, and thus build a driving force for water transport (Albrechtová and Wagner 2004). It is well known that aquaporins are involved in regulating water relations, and studies in *C. rubrum* revealed the expression of a novel aquaporin (CrAQP) with a transient increase in the expression a few hours before the maximal increase in the size of the meristem (Albrechtová and Wagner 2004; Albrechtová et al. 2004). It was

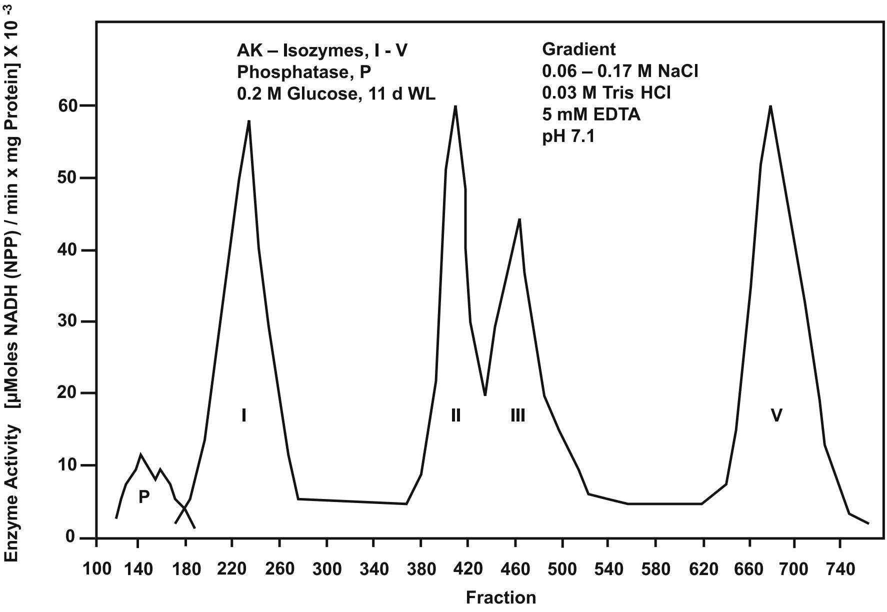

Fig. 25.7. Pattern of AK isozymes from *C. rubrum* (ecotype 374) after chromatography on DEAE-cellulose. Seedlings grown for 4.5 days at 12 h : 12 h 32.5 °C:10 °C and 6,500 lx continuous white light on filter paper with H_2O; thereafter on Hoagland's solution at 20 °C for 1 day followed by 5.5 days on 0.2 M glucose in Hoagland's solution. The abundance of the different isozymes in the cellular compartments changes after glucose application as compared with that in untreated plants (c.f. vegetative pattern as a control, Fig. 25.6). There is an increase of AK II and AK III abundance in the cytoplasm and the chloroplasts, respectively. AK IV (nucleus) was not detected (Wagner et al. 1983)

concluded that the change of aquaporin expression at the apical meristem during floral transition could be responsible for increased water movement into the meristem provoking its expansion. Further studies should reveal if intracellular pH and calcium concentration can influence water transport by regulating CrAQP activity (Lopez et al. 2003).

25.7 Electrophysiological Integration of Activity of the Whole Plant – Monitoring of Surface Sum Potentials

Automatic measurements of up to 4 weeks' duration could be performed using a measuring system with surface electrodes for the recording of surface sum potentials. The following measuring procedures were used (Wagner et al. 2004).

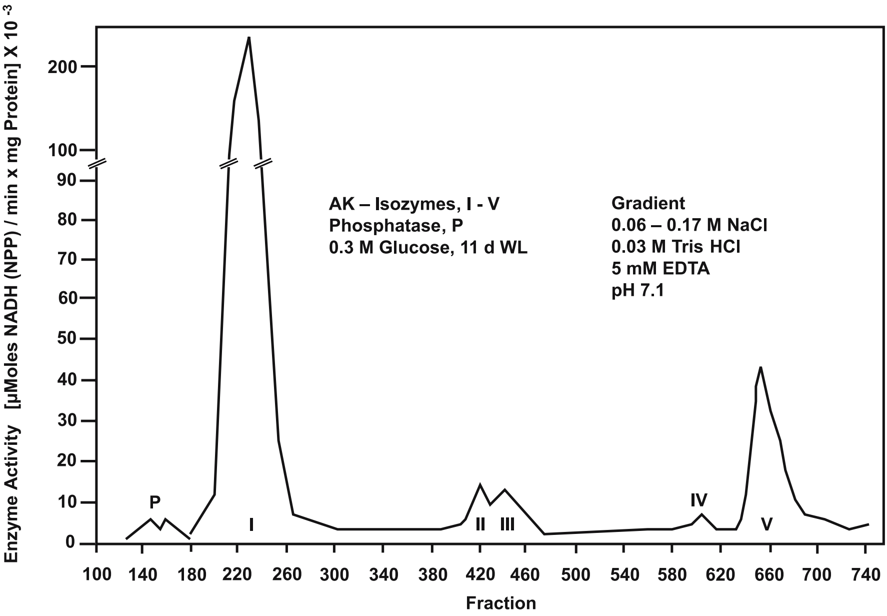

Fig. 25.8. Pattern of AK isozymes after chromatography on DEAE-cellulose. Growing conditions as in Fig. 25.7, however, with 0.3 M glucose application. There is a dramatic increase of AK I abundance in mitochondria as compared with that in untreated plants (c.f. vegetative pattern, control, Fig. 25.6). The abundance of AK IV in the nucleus increased over the detection limit (Wagner et al. 1983)

The electrodes essentially cover, i.e. surround, the stem axis or the petioles of leaves completely. This geometry of contact between surface electrodes and the plant tissue assures, in contrast to intracellular penetrating electrodes, that the potential changes to be recorded are not due to single cells but that they represent the electrochemical activity of the whole area of contact of the electrode with the plant tissue in question. For this reason the potentials recorded with surface electrodes are named "surface sum potential" or "surface potential". To reduce the resistance between the plant and the electrode surface, the contact surfaces between electrode and plant can be covered with a thin contact gel like one used in medicine, e.g. for recording electroencephalograms. The analysis or evaluation of the recorded changes in membrane potential are performed on the basis of the frequency, the temporal distribution and/or the direction of propagation of action potentials. In long-term experiments, using bipolar surface electrodes specific changes in the direction of propagation of action potentials in response to different flower-inducing and non-flower-inducing photoperiods could be observed. The recordings have shown that the propagation of action potentials along the stem axis of the model plants under investigation, *C. rubrum*

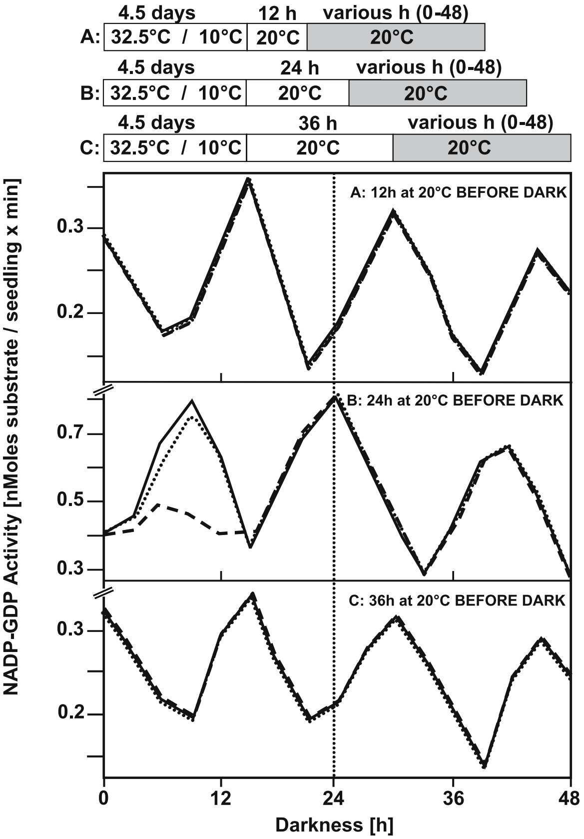

Fig. 25.9. Time course of NADP-GPD activity during darkness. *C. rubrum* seedlings germinating in alternating conditions of light intensity and temperature for 4.5 days. Thereafter constant 20 °C, 6,000 lx fluorescent white light for **A** 12 h, **B** 24 h and **C** 36 h followed by a dark period of varied duration (3-h increments). At the beginning of the dark period the seedlings received 5 min of red (*solid line*), 5 min of far-red (*dashed line*), or 5 min of far-red plus 5 min of red (*dotted line*) light (corresponding to a dusk signal in daily light–dark cycles), in order to modulate the level of active phytochrome. The enzyme activities were measured at the end of each respective dark period. The rhythmic germination conditions synchronise the endogenous circadian rhythm to exactly 24 h with an alternation of skotophile and photophile phases. The imposition of darkness after 12 and 36 h of constant conditions begins at the same phase, while the beginning of darkness after 24 h of constant conditions is 12 h out of phase with respect to the germination rhythm and to the other two treatment as is obvious from the semicircadian oscillations of enzyme activity in the three parts of the figure. The results demonstrate the different sensitivity to phytochrome amplitude modulation of enzyme activity oscillations depending on the phase of the underlying cycle of photophile and skotophile phases. Clearly the endogenous rhythm is gating the window for phytochrome action (Frosch and Wagner 1973)

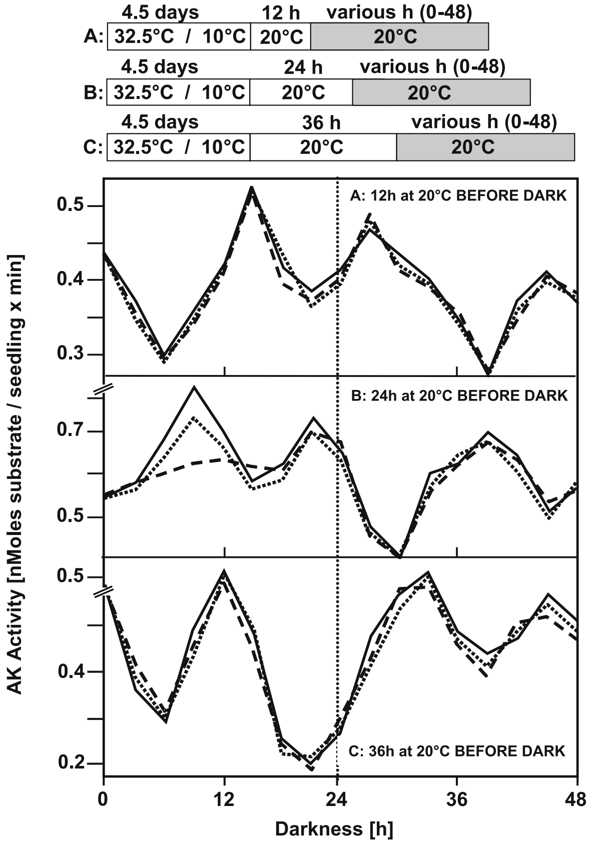

Fig. 25.10. Time course of AK activity during darkness. Experimental protocol as for Fig. 25.8 (Frosch and Wagner 1973)

and *C. murale*, depends on the photoperiod inductive or non-inductive for flowering. The statistic evaluation of the propagation direction of the action potentials could be used as a marker for the induction of flowering in short- and long-day plants. On the basis of these data, the state of flowering or flower induction of the plant can be determined long before the first morphological changes at the apical meristem become visible. Our results furthermore indicate that the action potentials under the influence of flower-inducing and non-flower-inducing photoperiods are temporarily not uniformly distributed in the dark and light phases of the different photoperiods, but that they follow a characteristic distribution pattern. To analyse the correlation between the photoperiodic induction of flowering as a specific state of the plant material, and the temporal distribution of action potentials and their direction of propagation, the observed action potentials had to be classified. Of particular relevance are signals which can

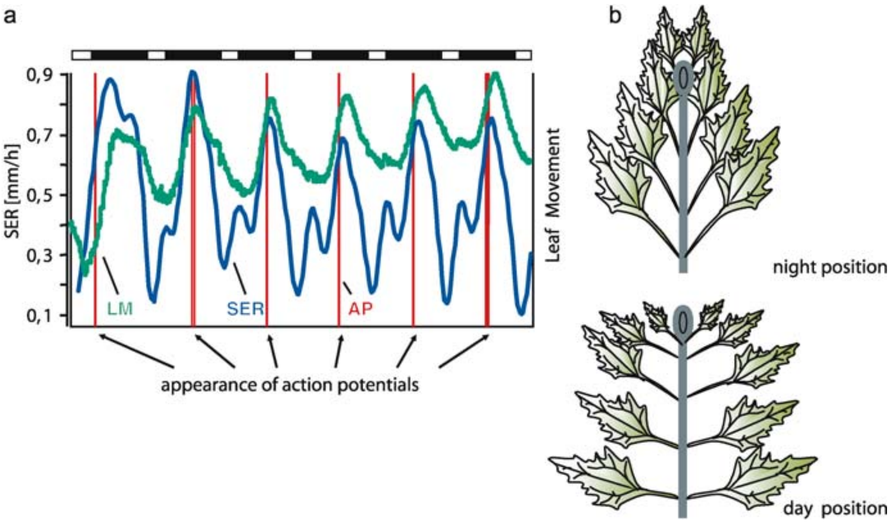

Fig. 25.11. **a** Kinetics of LM (*green line*), SER (*blue line*) (millimetres per hour) and time of appearance of action potentials (*APs*, *red vertical lines*) in *C. murale* (6-week-old plants) during exposure to non-inductive photoperiod with a light-to-dark ratio of 4 h : 20 h. **b** Schematic drawing of LM of *Chenopodium* showing the diurnal change of leaf position

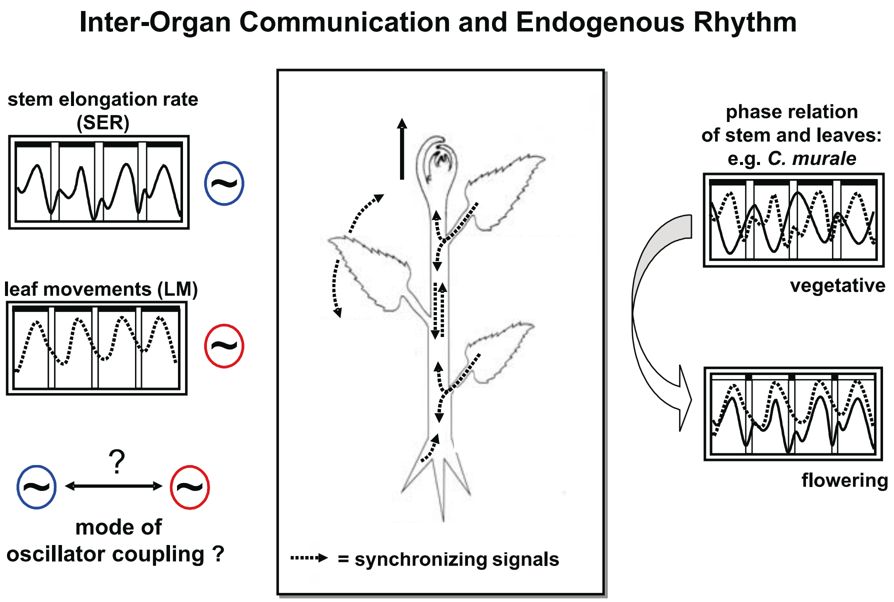

Fig. 25.12. Specific changes in the phasing of SER and LM kinetics appear upon flower induction. Both parameters depend on turgor-mediated growth and their interactions demonstrate rhythmic interorgan communication. LMs and SER are quantified by computer controlled time lapse photography (see Fig. 25.1)

be detected shortly after a light–dark or a dark–light transition (Wagner et al 1998). Measurements under the non-inductive light conditions for flowering did show that in the long-day plant *C. murale* mainly light-on signals were observed, while in the short-day plant *C. rubrum* an accumulation of the signals was observed after the light–dark transition. Under the influence of the flower-inducing photoperiods, both groups of plants showed an inverse pattern of action potential accumulation. In long-term measurements, the correlation between flower induction and the classification of action potentials either as light-on or as light-off signals could clearly be demonstrated. Therefore, this criterion is an important indicator for the state of flowering or for the differentiation between long- and short-day plant material. Thus, it has been shown that short-day plants and long-day plants each display characteristic distributions of action potentials during dark and light phases, which are characteristic for flower-inducing and non-flower-inducing conditions. It follows that the temporal distribution of action potentials over and within dark and light spans can also be reliably used as a marker for the flowering state of a given plant material (Wagner et al. 2004).

25.8 Substitution of Photoperiodic Flower Induction by Electrogenic Flower Induction

Specific electrophysiograms characteristic for photoperiodic flower induction were generated providing information concerning the electrical signal patterns of flower-induced plants. Experiments on electrostimulation using specific impulse patterns of DC current were aimed at inducing flowering under non-inductive light conditions. The results obtained showed that DC pulses during a fixed period of time for 1.5 h on seven consecutive days clearly induced seven of the 12 plants under investigation. With inverse polarity of the stimulating DC current, the apices of the stimulated plants did not show any difference compared to the apices of non-stimulated control plants (Wagner et al. 2004). These results clearly show that the adequate polarity of the stimulating electrodes is of decisive importance for flower induction. The temporal patterns of the stimulating currents should be selected depending on the type of plant (short-day or long-day) and the temporal pattern of the transition between endogenous oscillation in photophile and skotophile phases. The results obtained prove that flower induction in the plant can be induced by electrophysiological stimulation under non-flower-inducing environmental conditions.

25.9 Conclusions and Future Perspectives

The hydro-electrochemical communication of the higher plant is intrinsically linked with and depends on the membrane potential of cells and organelles, which can be modulated by the light environment (Trebacz and Sievers 1998). High-frequency (e.g. 24 min) temperature-compensated oscillations like in NADH oxidase activity of plasma membranes (Morré and Morré 1998) might be a high-frequency time scale for redox-control on a circadian scale. Populations of synchronised chloroplasts and mitochondria might be involved in the generation of proton-conducting structures like the mitochondrial network in animal cells (Giorgi et al. 2000). The hydro-electrochemical integration of the whole plant is the control net for integrating plant and environment in daily and seasonal adaptations using action potentials in a frequency-coded communication. The most important questions concern the mechanism of oscillator functioning in the various plant organs and the mode of coupling of oscillators, not to forget oscillator generation (Fig. 25.12). The following questions need immediate attention:

1. Where exactly are the action potentials for the systemic signalling generated?
2. What is the structural basis for stem polarity in the propagation of action potentials?
3. How does an induced leaf define propagation of action potentials in the stem?
4. What is the induced state of a leaf on the physiological level?
5. How does the photoperiod, e.g. via phytochrome (cryptochrome), create the induced state of a leaf?
6. What is common in an induced leaf from a short-day and a long-day plant?
7. How do circadian rhythmicity and hydro-electrochemical activity integrate the plant as a whole?

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26 Signals and Signalling Pathways in Plant Wound Responses

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Abstract The focus is on the most widely studied wound response: the systemic synthesis in tomato of the defence proteins known as proteinase inhibitors (PIs). In wounded tomato seedlings, the severity of the wound is a major determinant of the type of wound signalling. Minor wounds produce outcomes that are consistent with the transport in the phloem of a chemical elicitor of PI synthesis. Severe wounds produce outcomes that are consistent with the distribution of elicitors of PI synthesis and of electrical activity by hydraulic dispersal in the xylem. The main electrical events associated with severe wounds are action-potential-like depolarisations in the sieve-tube-element/companion cell complexes; these events are considered in relation to the use of the terms "action potential" and "variation potential".

26.1 Introduction

Plants respond to wounding both locally at the wound site and at sites distant from the wound (systemic responses). The most widely studied response is the systemic synthesis of defence proteins, known as proteinase inhibitors (PIs), in tomato seedlings following mechanical wounding, first discovered by Green and Ryan (1972). Mechanical wounding has been widely used to simulate insect damage. Our interest has been to elucidate the nature of the signals and signalling pathways underlying this systemic response. We believe that we can distinguish two situations: rapid signalling following a severe wound, and signalling in the phloem following a minor wound. The distinction between the effects of severe and minor wounds, in our view, enables a synthesis of the published results from several different research groups.

Several candidates have been proposed as a signal linking the local wound to the systemic response, including both chemical and physical signals, and a combination of the chemical and the physical: hydraulic dispersal. Given the complex nature of a wound, Bowles (1998) has highlighted the probability that such an event would give rise to multiple signals.

Possible chemical signals include oligosaccharides, jasmonic acid, and the peptide systemin; all have been shown to be elicitors of PI synthesis (Bowles 1998). Current evidence, based on mutations affecting PI synthesis, favours jasmonic acid as being a systemic signal, with systemin acting locally at or near the wound site – GA Howe's group, using minor mechanical

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wounding; see references in the review by Stratmann (2003). Following Baydoun and Fry (1985), it has generally been assumed that oligosaccharides act only locally, but the later work of Rigby et al. (1994) showed translocation in the phloem of an oligogalacturonide that induced PI synthesis. Thus, oligosaccharides remain as possible candidates for a systemic signal. Both oligosaccharides (Thain et al. 1995) and systemin (Moyen and Johannes 1996) produce transient membrane depolarisation, which could be a factor in the electrical activity associated with severe wounds.

Possible physical signals include a hydraulic pressure wave that arises when a wound releases the tension in the xylem of a transpiring plant. Such a pressure wave propagates through the plant from the wound site at high speeds, 100 mm s^{-1} or more; see the review by Malone (1996). A pressure wave could bring about its effects either by the activation of mechanosensitive (stretch-activated) channels (Stankovic and Davies 1997) or by the effect of signal molecules. Malone and co-workers showed, in tomato, that pressure pulses could be induced without significant wounding by submerged excision of a single leaflet through its submerged petiole; such a pressure pulse did not induce PI synthesis in tomato leaves. Instead, release of tension in the xylem sets up a reverse flow of xylem sap from a wound site into the rest of the plant, a phenomenon which they termed *hydraulic dispersal*; when this flow carried sap from wounded leaf tissue (e.g. a heat wound), systemic PI synthesis ensued, presumably owing to the presence of chemical elicitors in the sap.

Wounding also gives rise to electrical events that have usually been detected with surface-contact electrodes, and that appear to move through the plant from the wound site. These observations have led to the suggestion, e.g. Wildon et al. (1992), that the systemic signal for induction of PI synthesis is an electrical one, i.e. a self-propagating action potential similar to those in nervous conduction or in animal epithelial conduction: cell-to-cell conduction of action potentials (Mackie 1965). If that were true it should be possible to initiate the signal by depolarisation of the membrane potential of cells in the signalling pathway, preferably by injection of current via intracellular microelectrodes. It would also be expected that the electrical signals would show other characteristics typical of action potentials such as the all-or-nothing response, a strength–duration relationship for the initiating current, and a refractory period.

Evidence for the occurrence of action potentials in plants has been reviewed by Thain and Wildon (1996). There is clear evidence for action potentials in the charophyte algae, and in a number of higher plant species, which suggests that the ability to generate action potentials is widespread in the plant kingdom. Electrical stimulation has been used to induce PI gene expression (Herde et al. 1995; Peña-Cortès et al. 1995; Stankovic and Davies 1996, 1997), although intracellular electrodes were not used either

for the stimulus or for the recording, and the effect of heat generated at the plant–electrode interface cannot be discounted.

The wound-induced electrical events recorded with surface-contact electrodes are generally divided into two kinds (Mancuso 1999): those of irregular shape and of long duration (tens of seconds or more), which are generally called *variation potentials*; and those of simple shape (e.g. a single spike) and of shorter duration (a few seconds), which have generally been called *action potentials*. Clear examples of these two kinds can be seen in Fig. 1 of the paper by Stankovic and Davies (1996) but intermediate forms also occur. It must be emphasised that, for electrical events recorded with surface-contact electrodes, these terms are based purely on the shapes and durations of the electrical events; surface-contact electrodes record the sum, biased by the resistances of the various pathways between the cells and the surface, of all the membrane currents flowing in the underlying tissues. Unfortunately the terms variation potential and action potential are often taken to imply a mechanism. For example, it is widely assumed that variation potentials are the local electrical effects of chemicals flowing in the xylem stream. This could be true in many cases (Malone 1996), but it is also possible that an irregularly shaped event recorded with surface-contact electrodes could be the summation of a number of action potentials travelling along different pathways at slightly different times. Similarly the use of the term action potential to imply a mechanism is insecure in the absence of evidence from intracellular microelectrodes used to electrically stimulate and record from identifiable cells in the signalling pathway. At least for the electrical events following wounding, it may be best to avoid the variation potential/action potential terminology until a better understanding of the cellular nature of these events is obtained.

Obvious pathways for systemic chemical signals are the xylem and the phloem. A third would be the symplasm, but rates of transport, 15 $\mu m\ s^{-1}$, are too low for rapid systemic signalling. Rates of movement in both the xylem and the phloem can be high, with maximum reported speeds of 0.6–4.0 $mm\ s^{-1}$ in the phloem (Baker and Milburn 1989), and 250 $mm\ s^{-1}$ in the xylem (Passioura 1972). As already indicated, speeds for possible physical signals and hydraulic dispersal, are also high; thus, it may not be possible to use speed as a criterion to identify either the pathway or the mechanism. Several approaches can be used to distinguish between the various signalling possibilities: steaming of a short length of the pathway, followed by a recovery period, to disrupt the phloem but not the xylem; chilling to transiently block phloem translocation in some species, including tomato; the use of appropriate fluorescent dyes to visualise flows in the xylem or the phloem.

With regard to electrical signals, the phloem sieve-tube-element/companion cell (STE/CC) complex provides a suitable pathway with good

longitudinal connectivity, a functional plasma membrane, and low lateral connectivity with surrounding tissues (van Bel 2003). However the phenomenon of epithelial conduction in animals shows that action potentials can propagate through tissue consisting of relatively unspecialised cells, so it is not impossible that action potentials could propagate along other tissues, as long as they have adequate electrical connectivity via plasmodesmata.

We now consider the possibilities for signals and signalling pathways mainly in the light of results from our laboratory.

26.2 Patterns of Proteinase Inhibitor Activity and Electrical Activity Following a Variety of Wounding Protocols Applied to Tomato Seedlings

In our work (Rhodes et al. 1999) we used five different wounding protocols: (1) a razor cut through a cotyledon in air; (2) a razor cut simultaneously through a cotyledon and a droplet of Lucifer Yellow CH (LY) solution placed on its surface (i.e. cut under water); (3) a small (area about 60 mm^2) mechanical wound; (4) a large (area about 150 mm^2) mechanical wound; (5) a heat wound using a hot spatula held under, but not touching, the lamina. Wound-type 3 is referred to as a minor wound; wound-types 4 and 5 are referred to as severe wounds. For all the wounding protocols, we used surface electrodes on leaves 1 and 2 (Fig. 26.1) to check for electrical activity. Significant results are summarised in Table 26.1.

A razor cut through the lamina of a cotyledon in air (wound-type 1) produced neither electrical activity at leaf 1, nor systemic induction of PI activity (Table 26.1), as would be expected for a wound type in which neither a hydraulic pressure wave, nor hydraulic dispersal occurs.

A razor cut through a droplet of water on the lamina of a cotyledon (wound-type 2) induced systemic PI activity, but did not result in any electrical activity (Table 26.1), showing that electrical activity is not caused by reversed xylem flow alone. The pattern of induction of PI activity was striking: when cotyledon 1 (see Fig. 26.1, legend) was cut, more PI activity was seen in the right side of leaf 1 and the left side of leaf 2 (Fig. 26.1: *low* values indicate *high* PI activity). The pattern was reversed when cotyledon 2 was cut. To trace the pattern of flow in the xylem, the razor cut was made through a droplet of LY solution on the surface of the cotyledon. The flow of dye was observed in real time, and travelled with an average speed of 5 mm s^{-1}; petiole sections confirmed that the flow was in the xylem. The distribution of LY in leaves 1 and 2 depended on which cotyledon was cut:

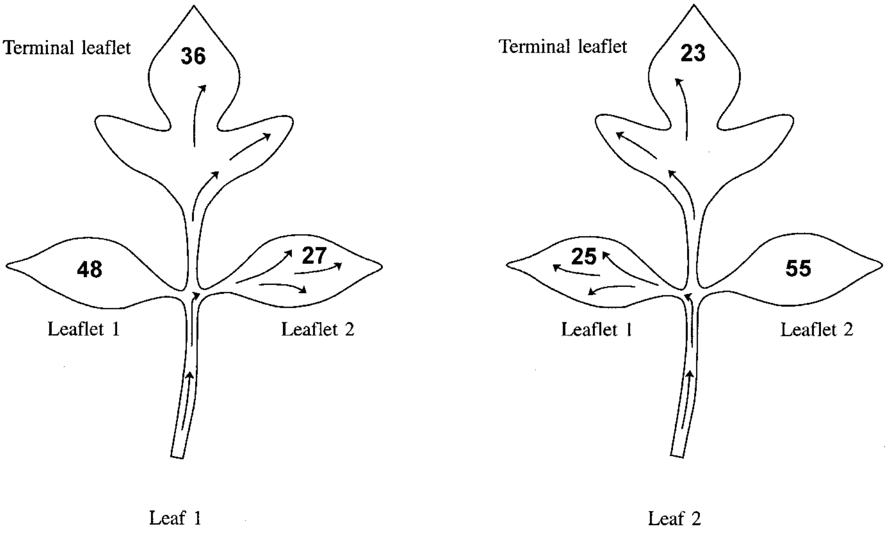

Fig. 26.1. The results of experiments with wound-type 2. *Numbers* show the pattern of the activity of chymotrypsin added to leaf extracts of *leaf 1*, the oldest true leaf, and *leaf 2*, and are expressed as a percentage of unwounded control plant samples; *low* values indicate *high* proteinase inhibitor activity. The *arrows* show the pattern of movement of the dye Lucifer Yellow CH (*LY*) from a droplet placed on the surface of the lamina of cotyledon 1 (the cotyledon facing the observer when the plant was positioned with leaf 1 pointing to the right), through which a clean cut was made using a razor blade. (Reproduced from Rhodes et al. 1999, with the permission of Oxford University Press, copyright of the Annals of Botany Company)

when cotyledon 1 was cut the LY entered the right side of leaf 1 and the left side of leaf 2 (Fig. 26.1); the pattern was reversed when cotyledon 2 was cut. The patterns of dye movement are directly linked to the architecture of the xylem. Thus, there was an observable reversal of flow in the xylem out of the wounded leaf, which could carry chemical elicitors to the rest of the plant. The distribution pattern of this hydraulic dispersal in the xylem coincided with the pattern of PI induction.

To track the movement of water in the xylem following other types of wounding, LY was injected into the lamina of a cotyledon, a procedure that did not cause systemic PI activity. Both a large mechanical wound (wound-type 4) and a heat wound (wound-type 5) when applied to injected cotyledon 1 caused uptake of LY by the xylem; flow of LY into leaves 1 and 2 followed the pattern already described for a cut across a droplet of LY on the lamina of cotyledon 1 (Fig. 26.1). The pattern was reversed when cotyledon 2 was injected and severely wounded. These severe wounds were accompanied by electrical activity, whose pattern coincided with the pattern of flow of LY. Severe wounds to cotyledons induced high levels of PI

Table 26.1. Summary of significant results. (Reproduced from Rhodes et al. 1999, with the permission of Oxford University Press, copyright of the Annals of Botany Company)

Wound type	Electrical event recorded with surface electrodes	Xylem reversal	Systemic proteinase inhibitor – no steaming of petiole of wounded leaf or cotyledon	Systemic proteinase inhibitor – after steaming of petiole of wounded leaf 1 – wound-types 3–5
Razor cut through cotyledon in air (1)	–	–	–	
Razor cut through cotyledon, under water (2)	–	+	+	
Small mechanical wound to terminal leaflet of leaf 1 (3)	–	–	+	–
Large mechanical wound to terminal leaflet of leaf 1 (4)	+	+	+	+
Heat wound to terminal leaflet of leaf 1 (5)	+	+	+	+

activity in all parts of the plant; the pattern of PI activity was less obvious than for wound-type 2, although where differences were found they always followed the pattern described before. Steam girdling the petiole of leaf 1 did not prevent the systemic induction of PI activity by a severe wound (wound-types 4 and 5) to the terminal leaflet of that leaf (Table 26.1); similar results were obtained by Malone and co-workers using heat-girdling. Following severe wounds, the patterns of PI activity, electrical activity, and the distribution of LY are consistent with the distribution of elicitors of PI synthesis and of electrical activity by hydraulic dispersal in the xylem; for such wounds, some involvement of the phloem is also possible (Rhodes et al. 1999).

Following a small mechanical wound (wound-type 3), in most cases, no LY flowed out of a cotyledon. LY was seen to flow out of leaf 1 following a large mechanical wound to its terminal leaflet, but not following a small mechanical wound to the same structure. Small mechanical wounds resulted in much lower levels of PI activity and a different pattern of distribution than those recorded for severe wounds or a razor cut under water: significant PI activity was found in the wounded terminal leaflet of leaf 1, and in leaf 3 and in the apex – organs which were identified, using carboxyfluorescein, as being active importers via the phloem, but very little PI activity was found in either of the cotyledons or leaf 2, which corre-

spondingly were not active importers via the phloem. These small wounds were not accompanied by electrical activity (Table 26.1). Steam girdling the petiole of leaf 1 prevented the systemic induction of PI activity by a small mechanical wound to that leaf (Table 26.1). These results are consistent with the report (Nelson et al. 1983) from Ryan's group that systemic signalling was prevented by prior hot air (80 °C) treatment of the petiole of the leaf that was to be wounded; the wounding protocol used by that group is, in our view, similar to wounding method 3, i.e. a small mechanical wound. We conclude that for small mechanical wounds the systemic signal is a chemical elicitor that is exported from the wounded leaf in the phloem.

Electrical events are associated only with severe wounds. Malone (1996) has provided evidence, on several grounds, that such electrical events are likely to be associated with hydraulic dispersal. On this basis, and from our evidence presented earlier for the similarities of the patterns of hydraulic dispersal (Fig. 26.1) and electrical activity, we conclude that the electrical events could be responses to chemicals transported in the xylem by hydraulic dispersal from the wound site, rather than action potentials propagated from that site. This is contrary to the conclusion in an earlier paper reporting work from our laboratory in which we used only severe wounds (Wildon et al. 1992); see also Davies (2004).

The conclusions here raise interesting questions about the results reported in our previous paper (Rhodes et al. 1996), which was the most detailed study to date of electrical events at the cell level in the wounded plant. In that work we used intracellular recording from all the cell types in the petiole of unwounded leaf 1 to further characterise electrical events following a severe wound (heat, wound-type 5) to cotyledon 1. The recording setup is shown in Fig. 26.2.

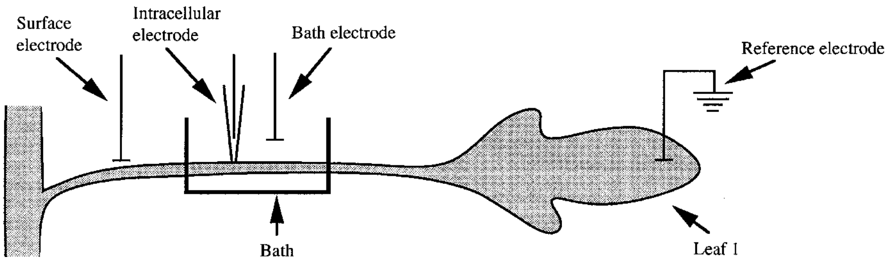

Fig. 26.2. Schematic diagram of the arrangement of electrodes on the petiole of *leaf 1* of a tomato seedling, used to record wound-induced electrical events. Each plant was wounded, using heat produced by passing an electric current for 30 s through a small wire element held over, but not touching, cotyledon 1 (Reproduced from Rhodes et al. 1996, with the permission of Springer-Verlag)

We obtained intracellular electrical recordings from the epidermal cells, cortical cells, phloem parenchyma cells, vascular parenchyma cells, and the STE/CC complex. The identification of the various cell types was achieved by iontophoretic injection of LY at the end of electrical recording. Figure 26.3 shows three of the cell types, and electrical recordings from these cell types: an epidermal cell (Fig. 26.3a,b), a cortical cell (Fig. 26.3c,d) and a STE/CC (Fig. 26.3e,f). Results from the other two cell types (phloem parenchyma cells, vascular parenchyma cells) were similar to those obtained from the cortical cells.

Only the phloem STE/CC complexes showed large electrical responses to wounding. These were large 'spike' depolarisations, which were very similar in shape and duration to known plant action potentials. The other four cell types showed only small depolarisations of longer duration, usually (except in the case of epidermal cells) with a small initial spike as is seen in the cortical cell record of Fig. 26.3d. It is possible that the small initial spikes seen with these other cell types are due to current from the large STE/CC spikes dissipating through surrounding cells via limited plasmodesmatal connections; visual comparison with the surface electrode and bath electrode traces suggests that the small spikes approximately coincide in time with the large STE/CC ones.

The large spike depolarisations seen in the STE/CC complexes are the main electrical events in the petiole following severe wounding. The origin of these spikes is not known. From their shape and duration they could be action potentials propagating along the STE/CC pathway. If they are action potentials, then presumably they have a function, but it would appear not to be the systemic signal for PI synthesis (Table 26.1). Alternatively they could be local responses to chemicals, as yet unidentified, but possibly oligosaccharides or systemin, carried in the xylem by hydraulic dispersal from the wound site. This latter possibility would lead to the following conclusions: firstly that, of all the cell types in the petiole, only the STE/CC complexes are sensitive to these chemicals; secondly that the STE/CC complexes have a mechanism for rapidly restoring their membrane potential to its normal value, presumably to protect them from the damaging effects of the large depolarisation. Without more detailed knowledge of the membrane events that cause these spikes, it is not possible to decide whether they are true action potentials.

While each experiment using intracellular electrodes allowed us to record the electrical events in only one STE/CC complex, these large depolarisations must have been happening in many of the STE/CC complexes in the petiole, either simultaneously or at slightly different times. The records from the surface and bath electrodes represent the summation of all the membrane currents flowing in the underlying tissues but, as noted before, these are primarily in the phloem STE/CC complexes. The shapes and du-

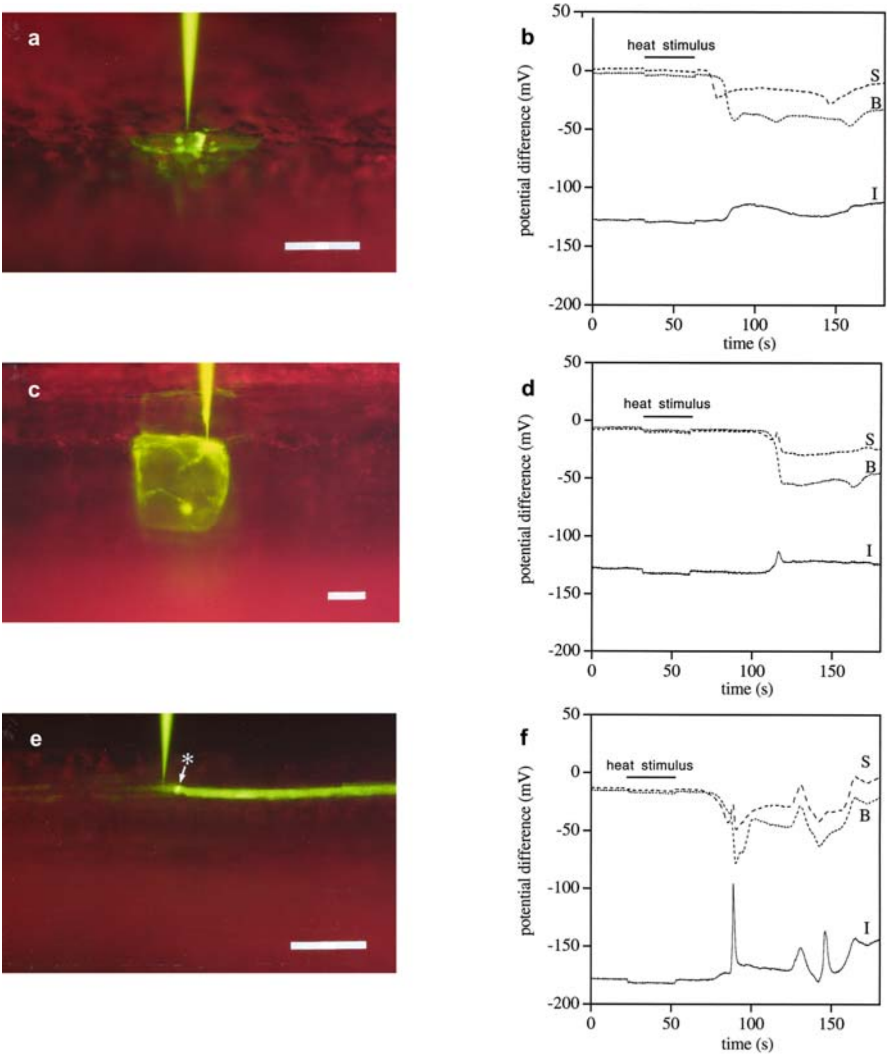

Fig. 26.3. Cells of the petiole of leaf 1 of 3-week-old tomato seedlings. **a,c,e** Cells labelled with the fluorescent dye LY via the glass micropipette used for intracellular recording: **a** an in situ epidermal cell; **c** an in situ cortical cell; **e** an in situ sieve-tube element: most of the dye has moved in the direction of the main stem, the *asterisk* indicates a sieve plate. *Scale bars* represent 100 μm. **b,d,f** Electrical recordings from the same cells as shown in **a,c** and **e**; **b** epidermal cell; **d** cortical cell; **f** sieve-tube element. *S* surface electrode (cotton thread soaked in 10 mM KCl connected to an Ag/AgCl electrode), *B* bath electrode (saline bath connected to an Ag/AgCl electrode by an agar/3 M KCl salt bridge), *I* intracellular electrode (glass micropipette back-filled with a 1% aqueous solution of LY followed by 3 M LiCl and an Ag/AgCl electrode). Heat was applied to cotyledon 1 for about 30 s causing wounding and a small artefact to appear on the traces (Reproduced from Rhodes et al. 1996, with the permission of Springer-Verlag)

rations of the electrical events recorded by the surface and bath electrodes would normally lead to their classification as variation potentials with the implication that they reflect depolarisations in the underlying tissues due to chemicals that have travelled in the xylem from the wound site. However, given our current ignorance of detailed mechanisms at the cell level, they could reflect the summation of action potentials travelling at slightly different times in the different STE/CC complexes of the petiole.

26.3 Conclusions and Future Prospects

For severe wounding, the results are consistent with the distribution of elicitors of PI synthesis and electrical activity by hydraulic dispersal in the xylem. We conclude that the electrical events are likely to be responses to chemicals transported in the xylem by hydraulic dispersal from the wound site, rather than action potentials propagated from the wound site.

Following a small crushing wound, the pattern of systemic PI synthesis was consistent with the transport of a chemical elicitor in the phloem from the wound site.

The lack of an electrical event following a small wound indicates that electrical signals are not an essential part of the systemic signalling system that induces PI synthesis.

The fact that a severe wound leads to large rapid action-potential-like depolarisations in the cells of the STE/CC complexes in the petiole, but not in other cell types, is intriguing. Do these depolarisations have a function?

In the absence of detailed studies at the cell level, the description of wound-induced electrical events recorded with surface-contact electrodes as either variation potentials or action potentials could carry misleading implications about their cellular mechanisms.

A significant step forward in plant signalling would be the identification of the cellular mechanism of wound-induced electrical events; this could be achieved using a similar approach to that described here and in Rhodes et al. (1996). However, since the mapping of its genome, *Arabidopsis* may be the preferred model organism; *Arabidopsis* does show electrical events following severe wounds (our unpublished observations). The identification of the ion channels responsible for the electrical events could be studied by the use of a range of agonists and inhibitors which could be introduced via the xylem stream and their electrical consequences in the phloem could be monitored using microelectrodes.

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27 Root Exudation and Rhizosphere Biology: Multiple Functions of a Plant Secondary Metabolite

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Abstract Plant root exudates have important effects on plant and soil communities, mediating positive and antagonistic plant–plant and plant–microbe interactions, and altering soil processes. Here, we discuss the potential for a single plant secondary metabolite to serve multiple functions in the rhizosphere. Secondary metabolites that serve multiple functions may provide plants with multiple benefits at relatively low metabolic cost, with consequences for competitive ability, disease resistance, and resource availability. Specifically, we describe recent research on (±)-catechin, a secondary metabolite exuded from the roots of the invasive weed *Centaurea maculosa*. Depending on concentration and the species that are present, (±)-catechin has the potential to act as an allelochemical, autoinhibitor, and antimicrobial agent, and to increase soil nutrient availability.

27.1 Introduction

Plants secrete a wide array of organic compounds into the soil surrounding their roots (i.e, the rhizosphere), including amino acids, carbohydrates, mucilage, and secondary metabolites (Bertin et al. 2003; Walker et al. 2003). The quantities of carbon released as root exudates are surprisingly large. Young plants (seedlings) may secrete over 30% of their photosynthates as root exudates (Sauerbeck et al. 1981), while older plants release between 5 and 21% of their photosynthates into the soil (Marschner 1995). Secondary metabolites, which are low molecular weight compounds produced during secondary metabolism, constitute the most diverse group of root exudates, and include an array of organic acids, flavonoids, tannins, terpenoids, alkaloids, polyacteylenes, and simple phenolics (Flores et al. 1999; Bertin et al. 2003). To date, more than 100,000 plant secondary metabolites have been identified (Dixon 2001).

Plant root exudates have important and diverse effects on soil chemistry, biology, and ecology. In particular, root exudates appear to mediate positive and antagonistic belowground communication between plants and their competitors, mutualists, and enemies. Secondary metabolites in root exudates are involved in the formation of symbiotic associations between plants and mycorrhizal fungi and bacteria (Dakora and Phillips 2002; Marx 2004). Other secondary metabolites in root exudates have been shown to possess phytotoxic, antimicrobial, antibiotic, insecticidal, and hormonal

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properties (Sudha and Ravishankar 2002; Bais et al. 2004, Bais et al. 2005), leading to inhibition of competitors, pathogens, herbivores, and parasites. Still other root exudates elicit herbivore defense responses in neighboring plants (Dicke and Dijkman 2001). In addition, root exudates can alter nutrient cycling, facilitate root movement, increase nutrient acquisition, and reduce metal toxicity (Dakora and Phillips 2002; Hawes et al. 2003).

The role of phytotoxic secondary metabolites in plant–plant interactions (i.e., allelopathy) has been the subject of considerable research and debate (Nilsen 2002; Bertin et al. 2003; Weir et al. 2004). Plants that produce and accumulate phytotoxins in the soil are thought to limit establishment, growth, and survival of neighboring plants, thus reducing local resource competition and increasing their own success. Potent phytotoxins have been found in plant leaf and root tissue, leaf leachates, leaf volatiles, and root exudates. However, because effects of phytotoxins on plant interactions are difficult to separate from effects of resource competition, many ecologists are not convinced that allelopathy plays an important role in plant communities (Fitter 2003).

In addition, the evolution of phytotoxic secondary metabolites in plants is not well understood, in contrast to the evolution of plant secondary metabolites that repel or attract insects and microbes (Ehrlich and Raven 1964; Whittaker and Feeny 1971; Scribner 2002). The selection pressures that could account for the development of phytotoxins in plants are relatively easy to imagine (e.g., reduced competition from neighbors). However, the selection pressures that might maintain the production of phytotoxic secondary metabolites over time are unclear (Fitter 2003). The argument that natural selection should operate against continued phytotoxin production is as follows. Allelopathic plants must be exposed to relatively high concentrations of their own phytotoxin. Consequently, to benefit from being allelopathic, a plant must be at least partly resistant to its own allelochemical. Further, the metabolic cost of resistance together with the metabolic cost of production must be low relative to the resource benefit of competitor inhibition. If an allelopathic plant is able to develop relatively cheap resistance to its own allelochemical, then other plants should also be able to evolve resistance at relatively little cost. Once other plants develop resistance, the competitive benefits of producing the phytotoxin should disappear. The cost of producing the phytotoxin once its benefits are lost should result in evolution of reduced production.

Examples of allelopathic plants that have become invasive when transported to new continents suggest that allelopathy may be particularly effective in novel habitats where native species have not had the opportunity to evolve resistance to the invaders' allelochemicals (Rabotnov 1982; Callaway and Aschehoug 2000; Bais et al. 2003; Vivanco et al. 2004). These studies emphasize the potential importance of allelopathy in novel plant interactions,

and the potential for the benefits of allelopathy to disappear over time as the competitors develop resistance. However, the selection pressures that lead to continued production of phytotoxins by allelopathic species in their native range, where their competitors have developed resistance, remain unclear. Plant–plant chemical interactions may be more complex than traditionally thought. For example, indirect effects of allelochemicals on soil communities may sometimes be more important to plant community composition than direct effects on plant growth or survival (Wardle et al. 1990; Inderjit and Weiner 2001). Such indirect modes of chemical interaction between plants have rarely been examined explicitly.

Recently, our research on phytotoxic plant root exudates has highlighted how single plant secondary metabolites can perform many functions within plant and soil communities, depending on concentration, the species that are present, and perhaps environmental conditions. The potential for single secondary metabolites to have numerous effects in the rhizosphere provides insights into the complex nature of the selection pressures that may act on secondary metabolite production and secretion. A secondary metabolite that can serve multiple functions may be produced initially for one purpose, and later for a different purpose. Further, the metabolic costs, and associated selection pressures, of producing a secondary metabolite may be substantially reduced relative to the benefits if the metabolite serves multiple functions. In this chapter, we will focus on one secondary metabolite, catechin, which is exuded as a racemic mixture of (+)-catechin and (–)-catechin from the roots of *Centaurea maculosa* (Lam.) (spotted knapweed), an exotic invasive weed in North American grasslands. Recent research indicates that (±)-catechin has the potential to act as an allelochemical, autoinhibitor, and antimicrobial and nematicidal agent, and to increase soil nutrient availability in *C. maculosa* communities (Fig. 27.1).

27.2
C. maculosa Invasion Ecology

C. maculosa is a tap-rooted, short-lived perennial, native to grassland steppes of central and eastern Europe. *C. maculosa* was accidentally introduced to northwestern North America in the late 1800s (Sheley et al. 1998). To date, *C. maculosa* has infested over 2.8 million hectares of North American grassland, mainly in the midwestern and western regions of North America (Müller-Scharer and Schroeder 1993). While *C. maculosa* is not particularly abundant in European grasslands, it is often the dominant plant in invaded North American grasslands (Ridenour and Callaway 2001). In North American infestations, *C. maculosa* densities can exceed

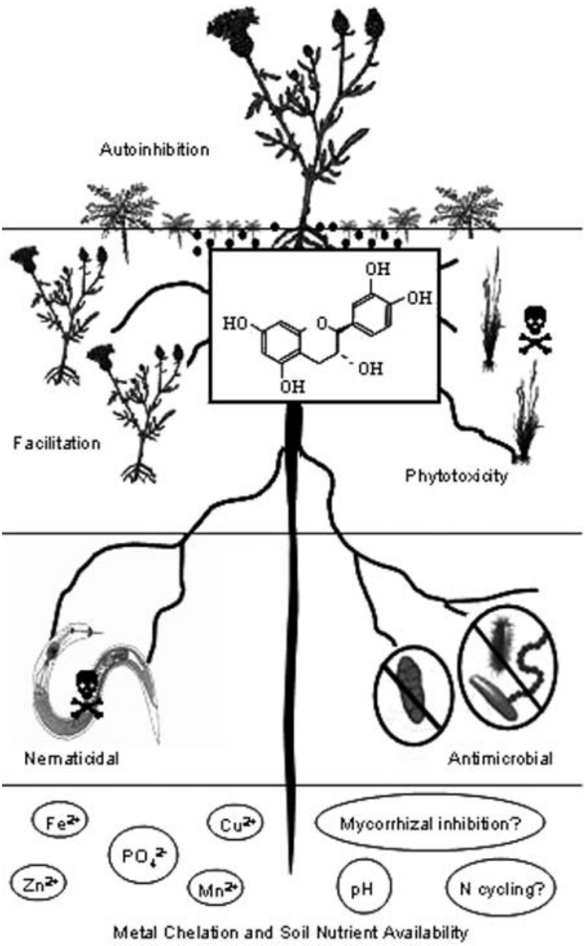

Fig. 27.1. A schematic depiction of the potential roles of (±)-catechin in *Centaurea maculosa* root exudates. (±)-Catechin is a potent phytotoxin with negative effects on survival and growth of interspecific competitors of *C. maculosa* (Bais et al. 2002, 2003). Reduced competitor growth owing to (±)-catechin exudation increases *C. maculosa* growth (Ridenour and Callaway 2001). Higher catechin concentrations lead to inhibition of *C. maculosa* seedling establishment (i.e., autoinhibition), perhaps serving as a mechanism for regulation of population density (Perry et al. 2005b). (+)-Catechin is antibacterial and antifungal, while (–)-catechin is nematocidal, perhaps leading to changes in pathogen and mutualist populations (Bais et al. 2002; Veluri et al. 2004b). Finally, (±)-catechin is a strong metal chelator with the potential to increase phosphorus and micronutrient availability (Callaway and Ridenour 2004). (±) Effects of catechin on nematode populations and perhaps mycorrhizal fungi may also alter nutrient cycling and other soil processes

400 individuals per square meter (Jacobs and Sheley 1998), and seed set can exceed 10,000 seeds per square meter (Schirman 1981). Monodominant stands of *C. maculosa* decrease plant biodiversity (Tyser and Key 1988; Ridenour and Callaway 2001), increase water runoff and soil erosion (Lacey et al. 1989), and reduce forage quality for livestock and wildlife (Watson and Reddy 1974; Thompson 1996).

Recent work on *C. maculosa* root exudates indicates that *C. maculosa* invasions in North America may be partly mediated by root exudation of a potent phytotoxin, (±)-catechin (Bais et al. 2003). *C. maculosa* invasiveness in North America probably results from a combination of factors, including allelopathy, high reproductive capacity (Schirman 1981), affinity for disturbance (Marcus et al. 1998), and competitive ability for limiting resources. *C. maculosa* does not appear to be a better competitor than North American species for nitrogen or water (Blicker et al. 2003; Olsen and Blicker 2003), but may be a better competitor for phosphorus in the presence of arbuscular mycorrhizal (AM) fungi (Zabinski et al. 2002). In addition, AM fungi may mediate carbon transfer from some North American grasses to *C. maculosa* (Carey et al. 2004). In the following, we describe the current knowledge of effects of (±)-catechin exudation on *C. maculosa* ecology and invasion.

27.3 (±)-Catechin, Allelopathy, and Cell Death

Evidence for allelopathy in *C. maculosa* was first reported in 1963 by Fletcher and Renny, who observed high concentrations of a phytotoxin, cnicin, in *C. maculosa* leaf tissue. However, cnicin concentrations in *C. maculosa* soils were found to be insufficient to inhibit establishment and growth of neighboring plants (Locken and Kelsey 1987), indicating that *C. maculosa* cnicin production was unlikely to be important for inhibiting plant competitors. More recently, Ridenour and Callaway (2001) tested whether *C. maculosa* root exudates might contain phytotoxins that inhibit growth of North American grassland species. They used activated carbon, which adsorbs organic compounds but has little effect on inorganic compounds (Mahall and Callaway 1992), to adsorb *C. maculosa* root exudates in the soil. Growing *C. maculosa* in soil with activated carbon reduced the inhibitory effect of *C. maculosa* on a native grass, *Festuca idahoensis* (Idaho fescue), suggesting that organic compounds in the soil around *C. maculosa* roots suppressed *F. idahoensis* growth. Further, the competitive effect of *F. idahoensis* on *C. maculosa* growth was 30% greater when activated carbon was added to the soil, suggesting that adsorption of *C. maculosa* allelochemicals shifted the balance of competition in favor of *F. idahoensis*. However, the

phytotoxins in *C. maculosa* root exudates needed to be identified to evaluate the role of allelopathy in *C. maculosa* dominance over North American grassland species.

27.3.1
Identification of the Allelochemical

To overcome the difficulty of isolating root-secreted allelochemicals from soil, Bais et al. (2002) grew *C. maculosa* in vitro and collected the root exudates in sterile media. Addition of crude *C. maculosa* exudates to liquid media where *Linaria dalmatica* (Dalmatian toadflax), *Verbascum thapsus* (common mullein), *Bromus tectorum* (downy brome), *Kochia scoparia* (kochia), *C. diffusa* (diffuse knapweed), and *Arabidopsis thaliana* were growing reduced root growth and induced plant mortality by 14 days after treatment, indicating that a component of the root exudates had broad-spectrum phytotoxic activity (Bais et al. 2002). The active fraction of the exudates was identified as (±)-catechin using high-performance liquid chromatography separations and identification by mass spectrometry and ^{1}H and ^{13}C NMR techniques. By testing commercially purchased isomers of pure catechin, Bais et al. (2002) initially attributed the phytotoxic activity to (–)-catechin. However, it was recently determined that (+)-catechin is also phytotoxic (Iqbal et al. 2003), although less potent than (–)-catechin (Veluri et al. 2004a).

27.3.2
Catechin Induces Reactive Oxygen Species and Ca^{2+}-Mediated Cell Death

As is the case for many allelochemicals, the exact cellular target of (±)-catechin is still unknown. However, many of the cell signaling and indirect molecular events that precede catechin-mediated cell death have been identified. Using real-time video imaging and fluorescent viability dyes, with *A. thaliana* and *C. diffusa* as target species, Bais et al. (2003) observed that cell death began about 15 min after catechin treatment, following condensation of the cytoplasm. Cell death occurred first in the meristematic zone of the root tip and moved upward to the central elongation zone, suggesting that catechin or catechin-induced signals are transported through the vascular system. To more fully understand the molecular basis for catechin-mediated cell death, Bais et al. (2003) monitored the kinetics of several cellular signals, including generation of reactive oxygen species (ROS), fluctuations in cytoplasmic calcium concentrations ($[Ca^{2+}]_{cyt}$), and

pH changes, to determine which signaling events occurred upon catechin exposure. ROS have been implicated in signal transduction events leading to downstream responses, such as modulation of gene expression, that affect several cellular and developmental processes (Huckelhoven and Kogel 2003; Finkel 1998). ROS are also known to accumulate in plant cells in response to compatible pathogen infections and may damage cellular structures and nucleic acids, resulting in cell death (Huckelhoven and Kogel 2003). Fluctuations in $[Ca^{2+}]_{cyt}$ are also known to occur in response to stress, including high salinity, osmoticum, heavy metals, and oxidative damage (Fasano et al. 2001; Jones et al. 1998). Finally, cytoplasmic pH changes are associated with signal transduction and regulation of root growth (Fasano et al.2001; Scott and Allen 1999), and cell viability is closely tied to homeostasis of cytoplasmic pH.

Bais et al. (2003) showed that all three factors play an important role in the initial events leading to catechin phytotoxicity. Using the fluorescent dye 6-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate-di(acetoxymethly ester), they determined that within 10 sec, catechin treatment induced a 12-fold increase in ROS generation in *C. diffusa* and *A. thaliana* seedlings. Like the pattern of cell death induction, the increase in ROS originated in the meristematic region of the root tip and moved into the central elongation zone before progressing back through the main axis of the root in a wavelike fashion. Catechin treatment also resulted in rapid, transient elevations in root-tip-localized $[Ca^{2+}]_{cyt}$ levels. These increases in $[Ca^{2+}]_{cyt}$ occurred about 30 sec after catechin treatment, following the ROS signal, but preceding cell death. Approximately 15–20 min after catechin administration, consistent with the initial appearance of cell death, the cytosolic pH dropped from approximately 7.2 to 5.6. These changes most likely reflect the loss of ion homeostasis associated with cell mortality. Bais et al. (2003) also demonstrated that exogenous application of an antioxidant, ascorbic acid, prevented the generation of the initial ROS signal, the subsequent spike in meristematic $[Ca^{2+}]_{cyt}$ levels, and cell death, suggesting that the phytotoxicity of catechin is directly or indirectly due to oxidative stress.

27.3.3 Catechin Exposure Leads to Genome-Wide Changes in *Arabidopsis*

Because the model plant *A. thaliana* is susceptible to catechin, Bais et al. (2003) were able to monitor changes in global gene expression to determine potential transcriptional events associated with catechin phytotoxicity. Using a 12,000 gene oligoarray, they monitored changes in transcription 10 min, 1 h, and 12 h after *Arabidopsis* roots had been subjected to phytotoxic levels of catechin. Within 1 h of treatment a number of genes related to oxidative stress were upregulated, including glutathione transferase,

monooxygenase, lipid transfer protein, heat shock protein, DNA-J protein, and blue copper-binding protein (Bais et al. 2003). Phenylpropanoid and terpenoid phytoalexin pathway genes, a number of which produce enzymes that can act as antioxidants (Sticher et al. 1997), were also induced in the roots 1 h after catechin treatment. However, the most interesting change in gene expression that they observed was the upregulation of ten genes in the first 10 min after catechin treatment. These included genes associated with calcium signaling and oxidative stress, as well as four unknown genes lacking homology with genes from other organisms. Identification of the function of these unknown genes could provide insights into the factors that cause plants to be susceptible to catechin, and into potential mechanisms of resistance.

27.3.4 (±)-Catechin Is Present at Phytotoxic Concentrations in *C. maculosa* Soils

While laboratory experiments demonstrated that (±)-catechin is a potent phytotoxin with strong effects on plant biochemistry and gene expression, field observations were required to gauge the importance of (±)-catechin in *C. maculosa* competitive interactions under natural conditions. In particular, for (±)-catechin to influence plant interactions, it must be present at phytotoxic concentrations in *C. maculosa* field soils. Several studies have reported exceptionally high soil (±)-catechin concentrations in North American *C. maculosa* soils (Bais et al. 2002, 2003; Perry et al. 2005b), indicating that soil (±)-catechin is present in sufficient quantities in *C. maculosa* soil to inhibit plant neighbors. Bais et al. (2003) reported a mean soil (±)-catechin concentration of 2.24 ± 0.20 mg g^{-1} and Perry et al. (2005b) reported a mean soil (±)-catechin concentration of 1.55 ± 1.27 mg g^{-1} dry soil. In addition, tests of the soil extracts from one site confirmed that the (±)-catechin in *C. maculosa* soils has similar phytotoxicity to (±)-catechin from commercial sources (Perry et al. 2005b). Bais et al. (2002) found that (±)-catechin concentrations declined with distance from the *C. maculosa* taproot, and with increasing soil depth. However, Perry et al. (2005b) found that soil (±)-catechin concentrations did not change with distance from the *C. maculosa* taproot but remained high as far as 25 cm from the taproot, indicating that high soil (±)-catechin concentrations may be ubiquitous in at least some well-established *C. maculosa* populations. The differences in soil (±)-catechin concentrations among the studies are not surprising, since the studies were conducted in different locations and at different times. The effects of soil characteristics, climate, and season on (±)-catechin secretion, stability, and soil absorption are not yet understood.

27.3.5 The Role of (±)-Catechin in *C. maculosa* Invasion

For (±)-catechin to facilitate *C. maculosa* invasion, it must inhibit the North American grassland species with which *C. maculosa* competes. Effects of (±)-catechin on seedling growth and survival have been examined for more than 25 North American grassland species that are native to the types of plant communities invaded by *C. maculosa* (Bais et al. 2003; Weir et al. 2003; Perry et al. 2005a). (±)-Catechin concentrations as low as 50 μg ml^{-1} reduce the growth of sensitive North American grassland species, such as *F. idahoensis* (Idaho fescue) and *Koeleria cristata* (prairie junegrass), grown in vitro in liquid media (Weir et al. 2003). Higher (±)-catechin concentrations (125–500 μg ml^{-1}) are required to significantly reduce the growth of sensitive North American species germinated on filter paper (Perry et al. 2005a), but these concentrations are still well below reported *C. maculosa* soil (±)-catechin concentrations. North American grassland species vary considerably in sensitivity to (±)-catechin (Perry et al. 2005a), indicating that (±)-catechin exudation probably gives *C. maculosa* an advantage over some species and not others. In particular, seedlings of larger-seeded species tend to be more resistant to (±)-catechin than seedlings of smaller-seeded species (Perry et al. 2005a). Some of the North American species that are relatively resistant to (±)-catechin appear to persist in *C. maculosa* populations, while others may be displaced by *C. maculosa* through resource competition rather than allelopathy.

One hypothesis to explain *C. maculosa* invasiveness in North America is that North American grassland species may be on average more sensitive to (±)-catechin than the European species with which *C. maculosa* naturally coexists, because the North American species have not had an opportunity to evolve resistance to the allelochemical (i.e., the "novel weapons" hypothesis; Callaway and Aschehoug 2000). To explore whether North American grassland species are more sensitive to (±)-catechin than European species, Bais et al. (2003) compared the effects of (±)-catechin on three North American grasses that are commonly displaced by *C. maculosa* and three European congeners that naturally coexist with *C. maculosa*. The North American species were significantly more inhibited by (±)-catechin treatment than the European species, suggesting that (±)-catechin may give *C. maculosa* a greater advantage over its competitors in North America than in Europe, probably contributing to *C. maculosa* invasiveness in North American grasslands.

27.4 (±)-Catechin and *C. maculosa* Autoinhibition

In addition to reducing the success of *C. maculosa*'s interspecific competitors, (±)-catechin appears to reduce *C. maculosa* seedling recruitment in well-established populations. In *C. maculosa* stands in North America, adult plants are sometimes widely spaced, with the interspaces between plants unoccupied, occupied by *C. maculosa* seedlings that fail to establish, or occupied by other species. Perry et al. (2005b) examined whether this pattern might be accounted for by (±)-catechin inhibition of *C. maculosa* seedling establishment (i.e., autoinhibition).

To test whether organic compounds in the soil in *C. maculosa* stands limited *C. maculosa* seedling establishment, Perry et al. (2005b) added activated carbon, which adsorbs organic compounds (Mahall and Callaway 1992), to the soil around adult *C. maculosa* plants in a well-established population in the field. Activated carbon addition increased *C. maculosa* seedling densities by more than 30%, indicating a strong negative effect of organic compounds in the soil around *C. maculosa* adults on *C. maculosa* seedling establishment. Seedling densities were still visibly greater in the soil with activated carbon than in the soil without activated carbon in some plots 1 year after the start of the experiment (L. Perry, personal observation), indicating that the effects of organic compounds in the soil around *C. maculosa* adults on *C. maculosa* seedling establishment are persistent.

Measurements of soil (±)-catechin around adult *C. maculosa* at the field site indicated that (±)-catechin concentrations in the site were extremely high (mean 1.55 mg g^{-1} dry soil). *C. maculosa* seedling densities at the site were very low (mean 28 seedlings per square meter). To test whether the high soil (±)-catechin concentrations could account in part for the low *C. maculosa* seedling establishment, Perry et al. (2005b) examined the response of *C. maculosa* seedlings to (±)-catechin in laboratory experiments. Treatment with 1.0 mg ml^{-1} of (±)-catechin reduced *C. maculosa* seedling root lengths by 50%, demonstrating that *C. maculosa* soil (±)-catechin concentrations are sufficient to substantially reduce *C. maculosa* seedling growth. Higher (±)-catechin concentrations (2 and 4 mg ml^{-1}) reduced *C. maculosa* root elongation by as much as 75%. Reduced root elongation did not result in seedling mortality under laboratory conditions, but would be expected to reduce survival under field conditions with limited water. In addition, when (±)-catechin was maintained in solution in 10% methanol for 2 days after treatment, treatment with 1.0 mg ml^{-1} of (±)-catechin reduced *C. maculosa* germination by more than 70%. Tetrazolium analyses of seed viability indicated that (±)-catechin treatment did not reduce seed survival, suggesting that (±)-catechin dissolved in methanol may have delayed germination of *C. maculosa* seeds.

Plant autoinhibition mediated by secondary metabolites has been the subject of much research (Singh et al. 1999). However, (±)-catechin is one of the first secondary metabolites determined to act as both an allelochemical and an autoinhibitor. Autoinhibition of seedling establishment may influence plant populations in several ways. First, autoinhibition may be a mechanism through which adults avoid establishment of intraspecific competitors in dense populations (Schenk et al. 1999). Second, seedlings that produce autoinhibitors may reduce intraspecific competition by preventing the establishment of their siblings and unrelated seedlings (Dyer 2004). Third, autoinhibitors that delay germination, rather than killing seeds or seedlings, may postpone germination in areas where intraspecific competition from adults would prevent seedling survival (Picman and Picman 1984). Determining the specific effects of autoinhibition on *C. maculosa* populations will require further research to determine whether (±)-catechin reduces *C. maculosa* seedling root elongation or delays germination under field conditions.

Autoinhibition by *C. maculosa* is probably most important in well-established stands, where soil (±)-catechin is likely to be concentrated and widespread. (±)-Catechin concentrations lower than 0.5 mg ml^{-1} have little effect on *C. maculosa* root elongation, and no effect on germination (Perry et al. 2005b). However, much lower (±)-catechin concentrations have large effects on other, more (±)-catechin-sensitive species (Bais et al. 2003; Weir et al. 2003; Perry et al. 2005a). Thus, *C. maculosa* seedlings should have an advantage over more (±)-catechin-sensitive species in soils with moderate (±)-catechin concentrations, while very high (±)-catechin concentrations may prevent establishment of *C. maculosa* seedlings as well as other species. In well-established *C. maculosa* populations with high soil (±)-catechin concentrations, autoinhibition, water stress (Jacobs and Sheley 1998), and seed predation (Muller-Schärer and Schroeder 1993) probably operate together to limit *C. maculosa* seedling establishment.

27.5 (±)-Catechin Effects on Soil Communities

In addition to the inhibitory effects of (±)-catechin on plants, (+)-catechin and (–)-catechin both reduce survival of some soil organisms. However, the two enantiomers appear to affect different organisms. Bais et al. (2002) found that (+)-catechin, but not (–)-catechin, inhibited growth of several soil bacteria. Six bacterial strains were exposed to each of the enantiomers or the racemic mixture, applied to filter discs. *Xanthomonas campestris* ssp. *vesicatoria*, *Erwinia amylovora*, *Pseudomonas fluorescens*, and *E. carotovora* were each susceptible to 100 μg ml^{-1} (+)-catechin or to 200 μg ml^{-1}

(±)-catechin. In contrast, *Agrobacterium radiobacter* was inhibited only by higher concentrations (300 μg ml^{-1} (+)-catechin or more), and *Agrobacterium rhizogenes* (15834) was not affected even by high (+)-catechin concentrations (Bais et al. 2002). None of the bacteria were affected by (–)-catechin treatment. Similarly, Veluri et al. (2004b) found that low concentrations of (+)-catechin (12.5–25 μg ml^{-1}) inhibited growth of several root-colonizing fungi, including *Tricoderma reesi*, *T. viridis*, *Fusarium oxysporum*, *Aspergillus niger*, and *Penicillium* sp. Veluri et al. (2004b) compared the antimicrobial activity of several catechin derivatives and found that (+)-catechin was the most active against *E. carotovora*, *E. amylovora*, *X. campestris*, and *A. radiobacter*, while the (+)-catechin derivatives (+)-pentaacetylcatechin, (+)-tetramethoxycatechin, and (+)-isopropylidylcatechin showed less antimicrobial activity. (+)-Pentaacetylcatechin and (+)-tetramethoxycatechin also inhibited some of the root-colonizing fungi. Notably, (+)-catechin antibacterial activity may be limited to gram negative species (Veluri et al. 2004b). (+)-Catechin has no effect or only a very weak effect on a number of gram positive human pathogens (Palma et al. 1999; Puupponen-Pimiš et al. 2001).

Although (–)-catechin does not appear to affect soil bacteria, (–)-catechin does appear to inhibit other soil organisms. Specifically, recent studies conducted in our laboratory have shown that (–)-catechin is nematicidal (B. Prithiviraj and J. Vivanco, unpublished data). Addition of low concentrations (1–10 g ml^{-1}) of (–)-catechin to the growth medium resulted in 100% mortality of the bacterial-feeding, saprophytic nematode *Caenorhbditis elegans* in 3–4 days. No previous studies have reported (±)-catechin inhibition of plant pathogenic or saprophytic nematodes. Such nematidical activity could have large effects on soil processes (discussed later) and on soil microbial populations. Free-living nematodes in the soil are often bacterial or fungal feeders, and thus could influence population dynamics of plant pathogens or mutualists.

In addition, soil fungi, and perhaps other soil microbes, may influence (±)-catechin secretion by *C. maculosa*. Fungal cell walls isolated from the soil-borne pathogen *Phytophthora cinnamommi* elicited increased root exudation of (±)-catechin by *C. maculosa* (Bais et al. 2002). *C. maculosa* plants grown together in vitro produced approximately 83.2 μg ml^{-1} of (±)-catechin in their pooled root exudates. When *P. cinnamoni* cell wall isolates were applied to *C. maculosa* plants in vitro, the (±)-catechin concentration in the root exudates increased to 185.04 μg ml^{-1}. The response of *C. maculosa* to fungal cell wall elicitors suggests that (±)-catechin may be an inducible plant chemical defense, similar to phytoalexins, that *C. maculosa* secretes in larger quantities when under attack by pathogenic microbes in the rhizosphere. Both constitutive and induced (±)-catechin exudation have the potential to affect the population dynamics of a wide

variety of rhizosphere microbiota under natural conditions. The influence of (±)-catechin on soil communities and the consequences for pathogen and mutualist abundances in *C. maculosa* soils warrant further investigation.

27.6
(±)-Catechin, Soil Processes, and Nutrient Availability

(±)-Catechin may also influence soil nutrient availability and nutrient cycling in the *C. maculosa* rhizosphere, through both direct chemical interactions with soil nutrients and indirect effects on soil communities (Callaway and Ridenour 2004). In laboratory experiments, catechin has been shown to be a relatively strong metal chelator, able to form stable complexes with iron, aluminum, and copper ions (Mhatre et al. 1993; Mira et al. 2002; Khokhar and Apenten 2003). Metal chelators in root exudates are thought to increase availability of soil micronutrients, including iron, manganese, copper, and zinc, by forming complexes with the metals and increasing their solubility and mobility (Dakora and Phillips 2002). Evidence that chelators in plant root exudates increase soil micronutrient availability is particularly strong with regard to graminoid secretion of phytosiderophores (Treeby et al. 1989; Cesco et al. 2002; Jones et al. 2004), but many phenolics, including catechin, produced by dicots also have the potential to form complexes with insoluble micronutrients and may have similar effects (Olsen et al. 1981; Dakora and Phillips 2002). In addition, metal chelators in root exudates are thought to increase availability of soil phosphorus, a frequently limiting macronutrient in terrestrial ecosystems. A large portion of soil phosphorus is often unavailable to plants because it is bound in insoluble ferric, aluminum, and calcium phosphates (Mengel and Kirkby 1987). Metal chelators, by binding to iron and aluminum in ferric and aluminum phosphates, release plant-available phosphates at the same time that they increase metal solubility (Masaoka et al. 1993; Dakora and Phillips 2002). Thus, (±)-catechin exudation may increase phosphorus and micronutrient availability in the *C. maculosa* rhizosphere, although effects of (±)-catechin chelation on nutrient availability have not yet been examined.

(±)-Catechin may also affect soil nutrient availability by altering soil communities. As described earlier, (±)-catechin is known to be toxic to some soil-borne pathogens and nematodes. The nematicidal effects of (±)-catechin could have profound effects on nutrient cycling. Laboratory experiments and field studies have demonstrated that nematodes play a critical role in influencing turnover of soil microbial biomass and nutrient availability (Bardgett et al. 1999; Bongers and Ferris 1999; Yeates 2003). In some ecosystems, nematode activity accounts for up to 40% of nutrient

mineralization as a result of nematode excretion and nematode effects on bacterial populations (De Ruiter et al. 1993). In addition, (±)-catechin in the rhizosphere may affect soil mycorrhizal fungi. Catechin concentrations in the roots of several tree species have been shown to decline in association with mycorrhizal infection (Münzenberger et al. 1990, 1995; Beyeler and Heyser 1997), suggesting either that tree roots reduce catechin concentrations to allow mycorrhizal infection to occur, or that mycorrhizal fungi use the catechin in infected roots as a carbon source (Beyeler and Heyser 1997). Several fungi have been shown to degrade catechin (Galiotou-Panayotou et al. 1988; Vasudevan and Mahadevan 1990). In either case, high soil (±)-catechin concentrations would be expected to influence the abundance of soil mycorrhizal fungi. (±)-Catechin exudation may increase the abundance of soil microbes that can survive high soil (±)-catechin concentrations, or that can use (±)-catechin as a carbon source, and reduce the abundance of those microbes that cannot. Such changes have the potential to alter nutrient mineralization, immobilization, and transformation rates, as well as mutualistic associations that affect resource availability (Laakso et al. 2000; Hamel 2004). Research on the effects of (±)-catechin on soil communities, particularly mycorrhizal communities, and on nutrient cycling is needed to determine the effects of *C. maculosa* (±)-catechin exudation on soil processes.

27.7 Conclusions and Future Prospects

In summary, (±)-catechin, a secondary metabolite exuded from the roots of *C. maculosa*, has been shown to mediate positive and antagonistic plant–plant and plant–microbe communication, with the potential for strong effects on (1) survival and growth of interspecific competitors, (2) conspecific seedling establishment, (3) survival of pathogenic and nonpathogenic soil organisms, and (4) soil processes. Given the numerous functions that (±)-catechin may have in plant communities, it will be difficult to determine the selection pressures that resulted in the evolution of *C. maculosa* (±)-catechin production and that continue to select for its production in its native and exotic ranges. It is likely that some or perhaps many of the potential effects of *C. maculosa* (±)-catechin production are unintended consequences of a compound produced for a particular function or combination of functions. More research will be needed to determine the field conditions under which (±)-catechin operates as an allelochemical, autoinhibitor, antimicrobial agent, or soil nutrition enhancer, and whether and when these functions might act in concert. Regardless, the myriad of roles that (±)-catechin may play in communities occupied by *C. maculosa*

highlights the importance of considering multiple functions in attempting to understand the effects and evolution of secondary metabolite production.

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28 Communication Between Undamaged Plants by Volatiles: the Role of Allelobiosis

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Abstract Plant–plant signalling is discussed from a tritrophic perspective, with special reference to results from experiments with a model system consisting of barley, aphids and ladybirds. Experimental support for the following statements is discussed: (1) barley plants communicate via volatile substances and, in certain combinations of genotypes, this communication leads to changes in biomass allocation, (2) communication between barley plants of certain genotypes changes the pattern of host plant acceptance by the bird cherry-oat aphid, *Rhopalosiphum padi*, (3) odour stimuli from barley and common weeds affect the searching behaviour of the seven-spotted ladybird, *Coccinella septempunctata*. The results indicate that an active response of the barley plant to exposure to weed *Cirsium arvense* volatiles may be involved. The tritrophic effects of plant–plant communication in barley add a new dimension to the term allelopathy. Thus, we use the term *allelobiosis* to denote interactions in which exchange of plant chemicals has an informative value for the receiving plant, and the response of the receiving plant affects its growth strategy, and relations with herbivores and their natural enemies.

28.1 Introduction

Coexistence with other plants is the commonest type of biotic challenge that an individual plant faces. With very few exceptions, plants have to stay where the propagation unit, such as a seed, happens to start its development. Thus, the survival of a plant depends on its capacity to meet challenges in the growing environment, including competition with neighbouring plants. One strategy for plants to deal with this may be to detect cues from their neighbours, and to respond in a way that reduces the negative effects of competition.

One type of chemical interaction between individual plants can take the form of 'chemical warfare', in which compounds that escape from one plant into the environment may affect the growth and development of neighbouring plants and their organs (Rice 1984). This was first described by Molisch (1937) who named it 'allelopathy' after the Greek *allelon* 'of each other', and *pathos*, 'to suffer'. The active compounds are usually non-nutritional chemicals whose effects on other plants depend upon their concentration and the environmental conditions. Allelopathy has long been an important issue in agricultural science, and has been shown to affect many aspects of

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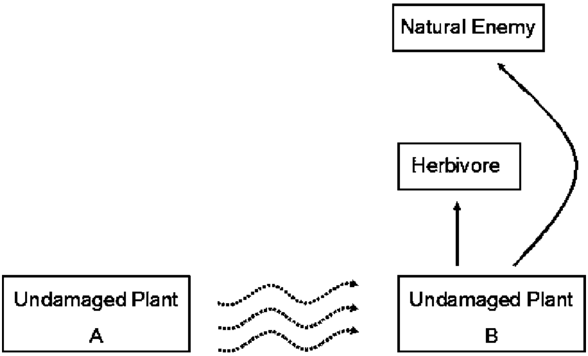

Fig. 28.1. Allelobiosis: the process of chemical interaction between undamaged pants and its effects on other trophic levels, e.g. insect herbivores and their natural enemies

plant coexistence and competition. However, from an ecological perspective, its role is still only partly understood and its general evolutionary importance is still a matter of speculation. For example, interactions of this type may affect not only the plant, but also herbivores and their natural enemies that are associated with the plant.

We have introduced a new term, *allelobiosis*, to describe the wider trophic effects of plant interaction via chemicals (Pettersson et al. 2003) (Fig. 28.1). The three key aspects of our definition of allelobiosis are (1) the chemical interaction occurs between undamaged plants, (2) the interaction may be beneficial for the receiving plant and (3) the responses of the receiving plant affect organisms at other trophic levels. Aspect 1 separates allelobiosis from a large body of research on interplant signalling, which focuses on signals released by infected/infested plants, while aspect 3 separates allelobiosis from the plant-focussed approach of allelopathy.

In theory, chemicals released by one plant may have an informative value for a neighbouring plant, and represent a stimulus that promotes changes in the growth strategy of the 'listening plant'. Potential effects on growth are changes in biomass allocation that, in the longer term, increase a plant's capacity to exploit resources such as light, water and soil nutrients. The altered growth strategy may also affect the physiological status of the plant, with implications for other organisms such as herbivores and their natural enemies.

In this review we explore the current knowledge of allelobiosis, and its effects across three trophic levels. Although allelobiosis can take place via several routes, including root exudates, we will focus here on plant volatiles. Special reference will be made to experiments on a model system consisting of barley, an aphid herbivore and a natural enemy, a ladybird.

28.1.1 Plant–Plant Communication via Volatiles – a Complex Language

Well-known examples of interactions mediated by volatiles from undamaged plants are the dominance achieved by shrub species (e.g. *Salvia leucophylla*, *S. apiana*, *S. mellifera* and *Artemisia californica*) through emission of chemicals such as α-pinene, β-pinene, camphene, champhor, cienole and dipentene that inhibit germination and root growth in seeds of herbaceous species (Muller et al. 1964). Methyl jasmonate produced by *A. tridentata* plants induced resistance to herbivores in leaves of neighbouring tomato plants by initiating the accumulation of proteinase inhibitors (Farmer 2001). Karban et al. (2000) showed that mechanically damaged *A. tridentata* plants increase production of methyl jasmonate and induce defensive responses to herbivores in wild tobacco, *Nicotiana attenuata*, although a recent study suggests that methyl jasmonate is not the active signal in this interaction (Preston et al. 2004).

There has been an explosion of interest in plant signalling in recent years, with a strong emphasis on volatiles emitted by plants in response to attack by herbivores or infection with pathogens. Such signals can induce a defence response in neighbouring, non-attacked plants, making them less attractive to herbivores. Studies have also shown that volatiles produced by plants induced in this way can promote searching behaviour of natural enemies of the herbivores. It is not our intention to review the entire spectrum of volatile plant–plant communication; however, a number of excellent reviews are available, including those by Bruin and Dicke (2001) and Farmer (2001).

28.1.2 Experimental Considerations in Plant–Plant Communication

Distinguishing the effects of plant interaction via chemicals from the effects of competition for resources has been the major hindrance to studies of allelopathy/allelobiosis in both laboratory and natural conditions. The first reports of communication between infested and uninfested plants (Rhoades 1983; Baldwin and Schultz 1983) met with some criticism focusing on the experimental design, particularly problems with pseudoreplication (Fowler and Lawton 1985). Pettersson et al. (1999) and Ninkovic et al. (2002) introduced a new method for plant exposure that separates allelobiosis from other interference mechanisms. The method is based on a large number of two-chamber cages, each of which constitutes an experimental replicate (Fig. 28.2). Pots containing the plants are placed in separate chambers, preventing competition for nutrients, light

Fig. 28.2. Twin-chamber cages for the exposure of barley plants to volatile chemicals from neighbouring plants. The chambers permit only interaction via plant volatiles, while eliminating root contact and competition for water and nutrients

and space. To prevent communication between roots, the pots are placed in Petri dishes (Fig. 28.2), or are grown in separate plastic bags placed under the bench (Ninkovic 2003). Air is drawn into the first chamber, where it collects volatiles from the inducing plant, it then passes through a hole in the wall to the second chamber containing the responding plant and is drawn out from the top of the second cage to a fan-equipped vacuum tank before being vented out of the room. A computer-controlled watering system simultaneously delivers precise amounts to the plants in all the cages.

28.2 Allelobiosis in Barley

28.2.1 Barley Plant Responses to Plant Volatiles

Studies on plant–plant communication in barley have mostly focussed on barley, *Hordeum vulgare*, as a responder to, rather than an emitter of, volatile cues. For instance, the growth of barley seedlings and the respiration rate of germinating seeds were inhibited when they were exposed to volatiles released from leaves of *A. tridentata* var. *vaseyana* (Weaver and Klovich 1977) and sasa, *Sasa cernua* (Li et al. 1992). These, and further, negative effects on barley plants, e.g. lowered content of water and chlorophyll, were observed in experiments with crude volatile oils and the pure terpenes from leaves of *Eucalyptus globulus* and *E. citriodara* (Kohli and Singh 1991).

Intraspecific allelobiotic interactions between plants have rarely been studied. In the case of interaction between barley plants, this phenomenon has been addressed in very few studies and usually from the viewpoint of induced resistance. Fujiwara et al. (1987) reported that volatile compounds released after pruning of barley leaves induced systemic resistance against powdery mildew fungus in exposed, intact barley seedlings. This resistance was more prominent in the primary leaf than in the secondary leaf. From the perspective of plant resistance to aphid, Pettersson et al. (1996) tested aphid acceptance of plants at the two-leaf stage that were exposed to volatiles from aphid-attacked plants or to powdery mildew-infested plants. In both cases aphid acceptance of exposed plants was significantly decreased in comparison with that of plants treated with clean air. The results of this study support a link between induced resistance to herbivores and disease in barley.

28.2.2 Allelobiosis and Plant Responses

In productive habitats such as fertilized arable land, similarities in resource requirements between individual plants of the same species intensify the struggle for capture of available resources. In the early stages of plant growth (seedling phase), shoots and leaves scarcely interfere with each other and competitive interactions, where they occur at all, are most likely to be limited to those operating within the soil. Plants can modify their growth in response to environmental conditions, allocating biomass to either aboveground or belowground organs in a way that maximizes growth. This trade-off between allocation to shoots and roots may be one of the plant's primary responses to competition with other plants (Grime 2001). If this is so, then it should benefit plants to detect competition not only through the depletion of resources, but by responding to the actual presence of potentially competing plant individuals. In this way plants could respond promptly to the presence of competitors, e.g. during seed germination or the early seedling phase, allowing them to minimize the negative effects of competition.

In a particular combination of barley cultivars, in which interaction between roots was prevented, seedlings responded to volatiles from neighbouring plants with changes in their pattern of biomass allocation (Ninkovic 2003). Plants exposed to volatiles from a different cultivar allocated more biomass to roots than did plants exposed to volatiles from the same cultivar, or to air alone. However, the total dry weight did not differ between treatments, and thus the principal effect was on the *allocation* of biomass, not on the *total* biomass (Fig. 28.3).

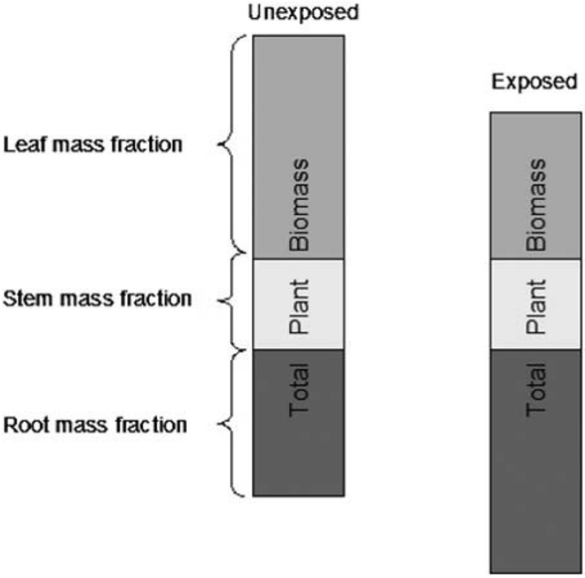

Fig. 28.3. Change in pattern of biomass allocation in barley after exposure of a plant of one cultivar to volatiles from a plant of a different cultivar. The exposed plant allocated greater biomass to roots compared with the unexposed plant, but total biomass was unchanged

Even though plants exposed to volatiles from a different cultivar allocated more biomass to roots than to shoots and leaves, their relative growth rate (RGR, increase in biomass per unit biomass per unit time) and unit leaf rate (ULR, increase in biomass per unit time and leaf area, a physiological component of the RGR) were not significantly different from that of either type of control plant. However, specific leaf area (SLA, leaf area per leaf dry weight), one of the two morphological components of RGR, was significantly increased in plants exposed to volatiles from the other cultivar. This is in line with previous studies showing that reduced biomass allocation to leaves can be compensated for by higher SLA (Aerst et al. 1991). It has been speculated that fast-growing plants benefit from a high SLA only if leaves increase their photosynthetic activity to the maximum (Van der Werf 1996).

Gersani et al. (2001) showed that individual plants sharing rhizosphere space with another plant produce more root mass than when one plant 'owns' that space. However, it seems that root growth can be stimulated merely by volatiles from a neighbouring plant, even though the plants do not share rhizosphere space (Ninkovic 2003). This indicates that allelobiotic responses affect the whole plant, not only specific parts such as roots or leaves.

In laboratory experiments, barley responses to allelobiotic interactions via volatiles from a different barley cultivar were measured in terms of changes in leaf temperature using an infrared camera (Pettersson et al. 1999). All possible pairwise combinations of four barley cultivars were tested. When certain cultivars were exposed to volatiles from certain other cultivars, significant reductions in leaf temperature were found.

Changes in leaf temperature result from active regulation of stomatal aperture transpiration, which depends on the water status of the plant modified by prevailing environmental conditions. Stomatal conductance, in turn, is a measure of transpiration. It has been reported that infrared measurements correlate well with data obtained using a diffusion porometer and with gas-exchange measurements, and studies have also shown local leaf temperature increases in regions where stomatal closure was induced by initial virus infection, before disease symptoms were visible (Chaerle and van der Streaten 2000).

Reduction in barley leaf temperature following allelobiosis indicates increased transpiration rates in exposed plants. It is probable that an increased need for water necessitates increased allocation of available biomass to roots in these plants. Higher stomatal conductance enhances the influx of CO_2, which is required to maintain a higher photosynthetic activity. The ULR is a physiological component of the RGR that is generally strongly correlated with the rate of photosynthesis (Poorter and Nagel 2000). To maintain the same level of the ULR, it seems that the photosynthetic activity of allelobiosis-exposed plants increased.

28.3 Allelobiosis and Insect Responses

Studies on the effects of plant–plant communication on insects have focused almost exclusively on interactions in which the responding plant is exposed to volatiles from herbivore- or pathogen-attacked plants. Volatiles produced by plants attacked in this way can induce responses in neighbouring undamaged plants, making them less attractive to herbivores (Bruin and Dicke 2001) and more attractive to the herbivores' natural enemies (Dicke and Van Loon 2000). Recent studies at the biochemical and genetic level have started to clarify the set of changes induced in responding plants by exposure to volatiles from herbivore- or pathogen-attacked plants (Arimura et al. 2000; Farmer 2001; Pickett et al. 2001). However, volatile communication between undamaged plants, and its implications for higher trophic levels, i.e. insect herbivores and their natural enemies, has been less studied.

28.3.1 Allelobiosis and Aphid Response

Aphids belong to widely distributed group of insects, of which numerous species are serious pests that damage plants mainly by sucking phloem sap, but also by transmitting plant viruses. Aphids make considerable use of chemical information in host plant location and selection, and are sensitive to changes in the quality and physiological status of their host plant (Pickett et al. 1992). Aphids feed by inserting a long flexible mouthpart, the stylet, directly into the phloem. Together, these factors make aphids an excellent model herbivore to detect changes in plants following allelobiosis.

28.3.1.1 Interspecific Allelobiosis and Aphid Host Plant Acceptance

When barley plants of certain cultivars were exposed to allelobiotic chemicals from common weeds (using the exposure system described earlier), they became significantly less acceptable to the aphid *Rhopalosiphum padi*. This occurred when barley was exposed to root exudates from the couch grass *Elytrigia repens* (Glinwood et al. 2003) and to volatiles from the thistles *Cirsium arvense* and *C. vulgare* (Glinwood et al. 2004). *R. padi* showed no behavioural response to volatiles from *Cirsium* spp. directly, and a range of compounds previously identified in *E. repens* root exudates did not negatively affect *R. padi* feeding on an artificial medium. Thus, exposure to allelobiosis from both weeds induced changes in barley plants that made them less acceptable for aphid feeding.

28.3.1.2 Intraspecific Allelobiosis and Aphid Host Plant Acceptance

When barley plants of one cultivar were exposed to volatiles from plants of a different cultivar (using the exposure system described earlier), they became significantly less acceptable to *R. padi*. This occurred only in certain pairwise combinations of four barley cultivars. This reduction of acceptability to the aphid also occurred when certain cultivars were exposed to volatiles *from the same cultivar,* i.e. self-exposure (Pettersson et al. 1999).

When the pairwise combinations were planted together in alternate rows in the field, certain combinations again led to reduced aphid acceptance of particular cultivars, compared with pure stands of that cultivar (Ninkovic et al. 2002) (Fig. 28.4). Whereas in the laboratory system plants interact only via volatiles, planting in the field allows for interaction via both volatiles and root exudates, as well as the effects of competition and environmental factors.

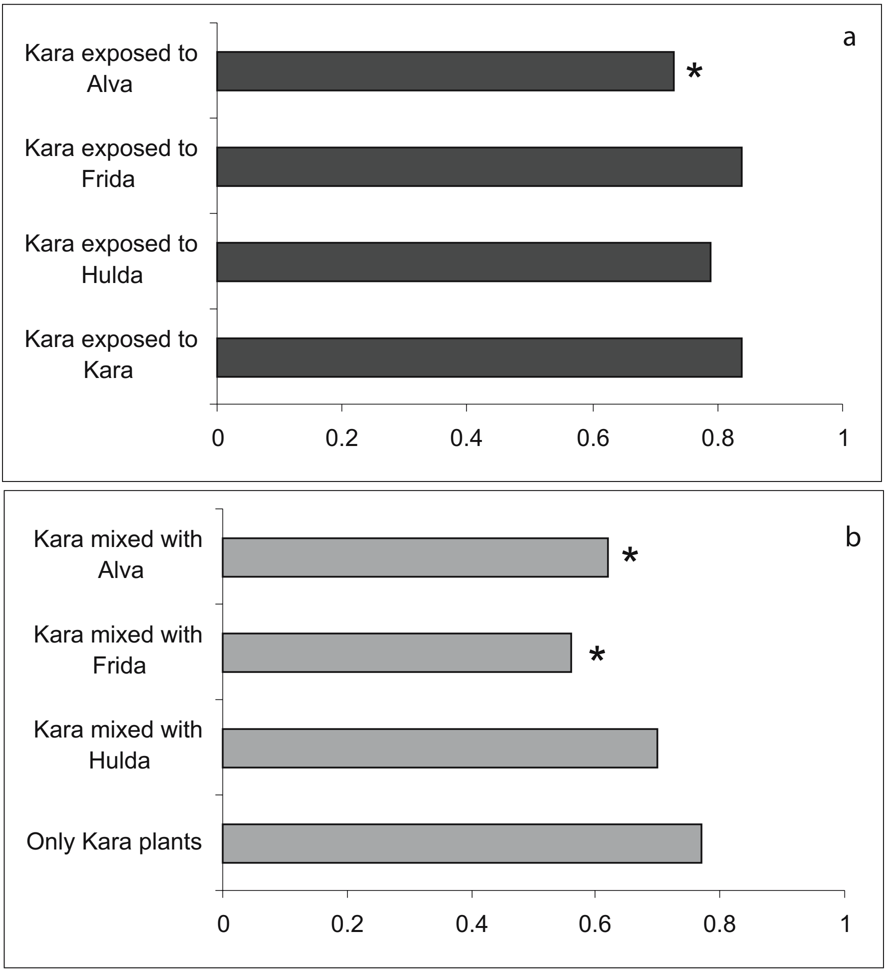

Fig. 28.4. Change in aphid plant acceptance of barley cultivar (Kara) **a** after exposure to volatiles from certain other barley cultivars in laboratory studies and **b** growing together with certain other barley cultivars in a field experiment ($^*P < 0.05$) (Ninkovic et al. 2002)

To our knowledge, effects on herbivores following aerial allelobiosis between undamaged plants of the same species have not previously been shown. The results indicate that the plant response is a genotype-regulated phenomenon that is dependent upon the characteristics of both the inducing and responding cultivars. Genetic variations in barley cultivar responses to weed-produced allelopathic compounds have been demonstrated (Ray and Hastings 1992).

28.3.1.3
Intraspecific Allelobiosis and Aphid Olfactory Response

Plant odour plays an important role for aphids in host plant location and selection (Pickett et al. 1992), and changes in volatile profiles can indicate plant physiological status. In olfactometer experiments, *R. padi* was significantly less attracted to the combined odour of two different barley cultivars that were allowed to interact via volatiles than to the combined odour from the same two cultivars that were isolated from each other (Pettersson et al. 2003). Odour from a plant of a single cultivar that had been previously exposed to volatiles from a plant of the other was also less attractive than odour from an unexposed plant.

This indicates that exposure to volatiles from a different cultivar may induce a systemic change in the plant that can lead to a modified volatile profile. This may be one factor responsible for reduced aphid plant acceptance of exposed plants.

28.3.2
Allelobiosis and Ladybird Searching Behaviour

The seven-spotted ladybird, *Coccinella septempunctata*, is an important aphid predator, but its impact as a control agent is variable. The searching behaviour of the seven-spotted ladybird has been studied from many perspectives (Dixon 2000), and food-searching behaviour of adults is influenced by volatiles emitted by barley plants infested with aphids (Ninkovic et al. 2001).

It seems to be a general phenomenon that increased botanical diversity reduces the incidence of pests, and enhances the impact of their natural enemies. So far the effects of aerial allelobiosis between undamaged plants sharing an environment have not been considered as a factor that can contribute to this phenomenon. In a field study, the abundance of adult *C. septempunctata* was greater in barley plots containing high naturally occurring densities of the common weeds *C. arvense* and *E. repens* than in other plots containing only barley (Ninkovic and Pettersson 2003).

In a subsequent laboratory study, adult *C. septempunctata* showed significantly stronger attraction to mixed odours of barley and each of the two weeds, than to barley alone. Ladybirds responded differently to barley plants that had been previously exposed to volatiles from the two weeds. The barley plants that had been exposed to *E. repens* lost their attraction when *E. repens* was removed, whereas barley plants that had been exposed to *C. arvense* remained attractive even after *C. arvense* was removed. This indicates that volatiles from these weeds can induce effects in barley plants that affect habitat-searching behaviour by ladybirds.

28.4 Conclusions and Future Prospects

The work reviewed here shows that chemical communication takes place between undamaged plants, supporting aspect 1 in our definition of allelobiosis (see "Introduction"). It appears that this communication affects not only the plant itself, but also organisms at higher trophic levels, namely insect herbivores and their natural enemies (aspect 3). It also raises the idea that a barley individual may benefit from the communication (aspect 2) by detecting neighbouring plants via the airborne volatiles they produce. It is most relevant to discuss this latter aspect in the context of the results on plant biomass allocation.

In experiments with two barley cultivars, Ninkovic (2003) showed that when plants of a particular barley cultivar were exposed to volatiles from a different cultivar the exposed plant changed its pattern of biomass allocation, allocating more biomass to roots and less to leaves (Fig. 28.3). This pattern did not arise when plants were exposed to volatiles from the same cultivar (self-induction). This selectivity may be due to the presence or absence of specific substances in the volatile blends, but this is not necessarily the case. It is important not to overlook the possibility that the amounts of trivial plant compounds or the specific ratios between some of these might constitute the active signal. This presents a challenge to the chemical identification of the signal mechanism itself, but clearly this has high priority for study since increased knowledge in this area will lead both to greater understanding of the ecological role of allelobiosis as well as to possible applications in crop management.

Even though plants of the exposed cultivar modified their pattern of biomass allocation, both the total biomass and the RGR were not significantly different from those of unexposed plants. Further, exposed plants did not undergo any alteration of physiological activity compared with unexposed plants. Here it is interesting to speculate whether, in the barley model system, volatiles from a different cultivar represent a stimulus that mobilises a morphological plasticity in the exposed cultivar that allows it to respond to potential competition from a neighbouring plant. In this scenario, allelobiosis would represent a source of information for the responding plant.

Allelobiosis causes changes in exposed barley plants that make them less suitable for aphid settling than unexposed plants. From the perspective of the responding plant, allelobiosis can be viewed as a route for obtaining information on plant competitors, which should give an advantage. Alternatively, it can be considered as a chemical disturbance that is detrimental. For the herbivore, however, it should be advantageous to detect the changes in plant status associated with *either* situation, if these make the plant less

suitable as a host. Aphids are well known for their capacity to detect and use chemical indicators of plant condition, and can detect barley plants that are engaged in allelobiosis with neighbouring plants, via volatile cues before contacting the exposed plant itself. Owing to their sophisticated host selection behaviour and mechanism of feeding, aphids represent an excellent indicator of allelobiosis effects in plants, and future work will focus on identifying the changes that occur in exposed plants, and their importance for the herbivore as well as the plant itself. Ladybirds are polyphagous predators, representing the third trophic level in the barley crop system. The experimental results indicate that they respond positively to volatiles released by barley exposed to allelobiosis from other plant species. This supports the idea that the habitat-searching behaviour of adult ladybirds is influenced by indicators of plant status, and contributes to understanding of the mechanisms behind the effects of habitat diversity on natural enemies of pests in managed systems.

Ecological and evolutionary understanding of allelobiosis is still at a very early stage, and further knowledge of the potential benefits and costs to both emitting and responding plants is necessary to understand the importance of plant interactions of this type in natural and managed habitats. Deeper understanding of how plants receive and translate volatile signals will lend a fascinating perspective to research on the signalling systems involved. The most exciting question will be to what extent allelobiosis represents a source of information to the listening plant.

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